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LACK OF CORTISONE EFFECT IN THE EARLY STAGES OF INFLAMMATION AND REPAIR *

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This report presents certain interesting and, we believe, hitherto unknown features of the early tissue responses to injury in cortisone-treated animals. The depression and inhibition of the inflammatory and reparative phenomena following injury during treatment with cortisone or ACTH have previously been reported from this laboratory,[1,2] and have been confirmed by others.[3-5] Recently we have recognized that this inhibitory effect is not immediate, but follows a lag of between ½ day and 4 days during which the morphologic changes of injured tissues in cortisone-treated animals are essentially identical with those observed in controls.

EXPERIMENTAL FINDINGS

In experiments employing rats, the animals weighed between 180 and 220 gm. and were pre-treated with 15 mg. of cortisone acetate intramuscularly daily for 3 days before injury and daily thereafter until sacrificed. Control animals received an equal volume of the suspending medium.

Incised Wounds. Incised wounds of skin and subcutaneous tissue, produced aseptically in rats, were studied histologically after 24 hours. The extent of tissue edema in dermis and subcutaneous tissue, polymorphonuclear leukocytic infiltration, and hyperemia were similar to those seen in control rats. The swelling of the endothelial cells lining

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the small blood vessels was, however, minimal in the cortisone-treated animals.

At 48 hours, the difference between cortisone-treated and control animals began to appear, represented by a decrease in intensity of the vascular and exudative changes in the former, together with a more or less complete lack of fibroblastic and angioblastic proliferation and histiocytic migration. At 5 days, the controls always showed complete healing, while the cortisone-treated animals presented the previously described inhibition of connective tissue repair. Epithelization, however, appeared to occur normally (see discussion).

Reaction to Sterile Irritating Foreign Bodies. Pledgets of surgical gauze were introduced through tunnels into the subcutaneous tissues of rats on the same regimen of cortisone acetate. The findings at 24 hours were essentially like those described for incised wounds, namely, edema and cellular infiltration which were similar in the cortisone-treated animals and in the controls. Six days after the gauze had been inserted, the difference between the cortisone-treated rats and the controls was striking. Foreign body giant cells and angioblastic and fibroblastic proliferation were marked in the controls while in the cortisone-treated animals no foreign body giant cells, relatively few new blood vessels and little fibroplasia were seen. Grossly, also, the difference was striking since the gauze was firmly adherent and enmeshed in the tissues in the control animals but lay free in the tunnel in the cortisone-treated rats.

Reaction to Intradermal Injections of Bacillus coli Filtrate. Rabbits weighing between 2.5 and 3.5 kg. were treated with cortisone acetate on schedules as outlined later. Controls received an equal volume of the suspending medium. Sections of skin and subcutaneous tissue during the first 2 hours following the injection of *B. coli* filtrate showed marked edema, vasodilatation, migration of polymorphonuclear leukocytes and occasional mononuclear cells, and margination of leukocytes. We were unable to differentiate qualitatively or quantitatively between controls and rabbits pre-treated for 48 hours with daily doses of 25 mg. of cortisone acetate.

Experimental Fractures. Comminuted fractures of the femur were produced in rabbits with a technique described elsewhere.[2] Up to the fourth day following the trauma, it was not possible to demonstrate histologically an appreciable difference between controls and animals treated with 25 mg. of cortisone acetate daily for 3 days before fracture and daily thereafter until sacrifice. Edema, mild inflammatory infiltration, necrosis, and early fibroblastic proliferation were present in both groups, and quantitative differences were not obvious. In three

cortisone-treated animals, however, we believed that metachromasia of the injured tissues was less in amount and more patchy. From the fifth day on, the control animals showed increasing formation of osteoid and cartilage, while this differentiation of the callus was almost absent in the cortisone-treated rabbits, at least up to the eighth day following the fracture.

It is possible that the longer lag in these fracture experiments may be related to repeated traumatic injuries. The fractures were not immobilized and, during active motion of the animals, the multiple sharp bone fragments could lead to a more extensive and repeated destruction of cellular elements.

Injections of Vaccinia Virus in Rabbits. Vaccinia virus was injected deep into the corium in two groups of rabbits.[6] One group served as a control, the other received 10 mg. of cortisone acetate intramuscularly daily for 2 days before injection of virus and daily thereafter. Two control and two cortisone-treated animals were sacrificed at 6, 12, 24, and 48 hours and at 7 days after inoculation of the virus. At the 6-hour interval control and cortisone-treated rabbits showed histologically at the site of inoculation the same degree of infiltration with, and vascular margination of, leukocytes. At 12 hours margination was less pronounced, and at 24 and 48 hours leukocytes were markedly decreased in the cortisone-treated animals. The lesion at 7 days in the control animals was a typical pockmark with dissolution of tissue, while relatively no loss of tissue occurred in the cortisone-treated animals.

Discussion

Michael and Whorton[7] have reported observations in which differences have been detected between control and cortisone-treated animals from the beginning of the inflammatory reaction. The differences between their findings and ours are difficult to explain but may be related to the type of the inflammatory stimulus or to some other unknown factor. One may speculate, however, that the initial injury—whether mechanical, chemical, viral, or bacterial—by causing an immediate destruction of cells, brings about the release of substances capable of increasing vascular permeability (edema), the migration of leukocytes, and the dilatation of blood vessels. Under normal conditions this initial event starts a series of chemical changes which are necessary for the further stages of inflammation and repair. Perhaps these chemical changes do not take place under the effect of large doses of cortisone. This hypothesis is suggested by certain facts which have emerged during our studies. Metachromasia of the ground substance of the injured mesenchymal tissues is generally reduced and

its appearance delayed in cortisone-treated animals, suggesting that under such circumstances the mucopolysaccharides of the connective tissue do not undergo the same chemical changes as in untreated injured animals. When epithelialization occurs in incised skin wounds of cortisone-treated animals, a narrow zone of fibroblasts is generally seen immediately beneath the basal layer of the regenerated epithelium, but not in deeper areas. This suggests that in this location, but not elsewhere in the injured area, conditions are favorable for the proliferation of fibroblasts. These conditions may perhaps be related to the mucopolysaccharides that are said to be produced by the regenerating squamous epithelium in the region of the basal membrane.[8]

Conclusions

The delay in the appearance of elements of the inflammatory and reparative processes during the administration of cortisone is not apparent histologically in the earlier phases of these processes. Delay becomes evident only after a lag of from ½ to 4 days following application of various types of injurious stimuli, mechanical, chemical, and viral. It is suggested that mucopolysaccharides do not undergo the same chemical changes in cortisone-treated animals as in untreated controls, and that, as a result, the chemical factors favorable for the post-initial phases of reparative reaction are deficient.

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ILLUSTRATIONS

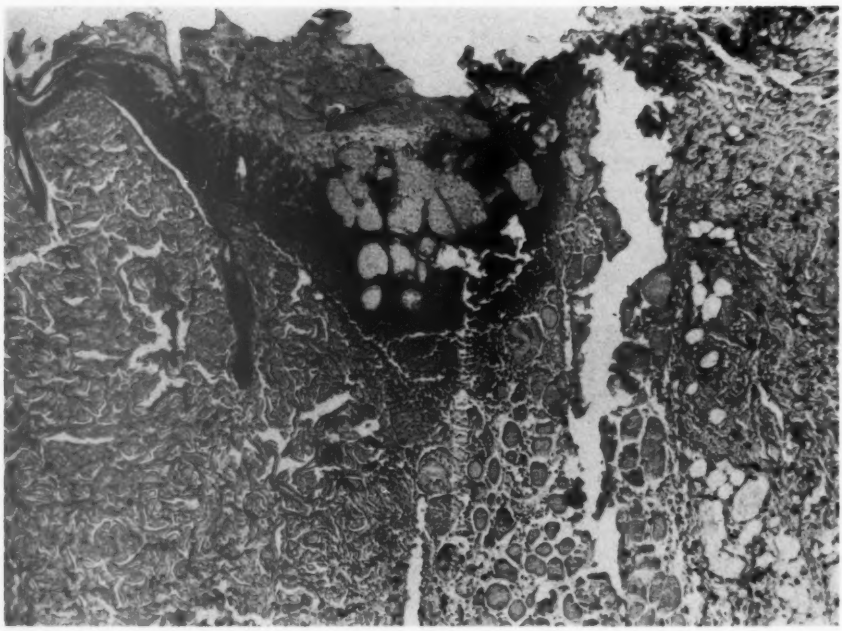
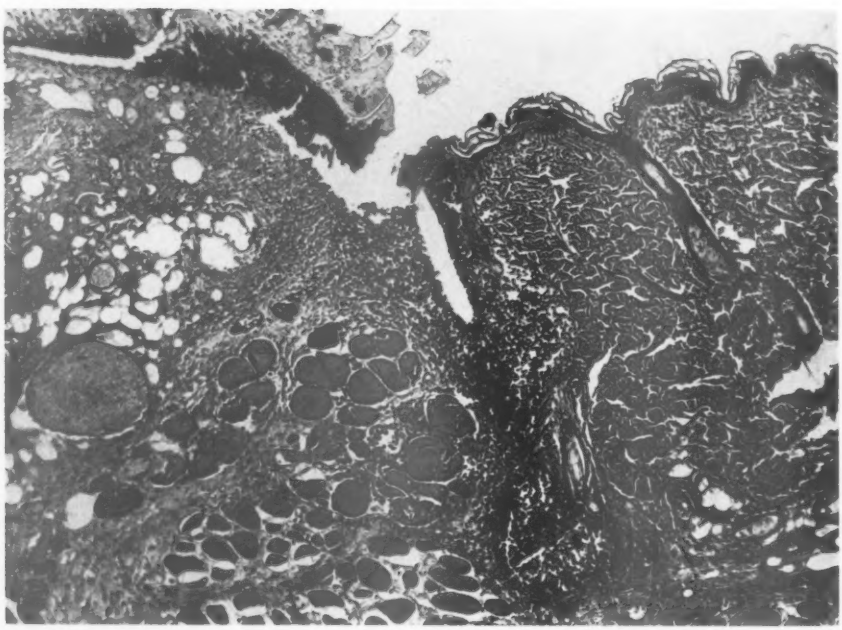


FIG. 1. Rat 13C, control, 24 hours after experimental production of incised wound in back. Fairly well marked edema and polymorphonuclear leukocytic infiltration along the margins of the wound may be seen. Hematoxylin and eosin stain. × 97.

FIG. 2. Rat 14E, cortisone treated, 24 hours after production of incised wound in back. Moderate edema and marked polymorphonuclear leukocytic infiltration are seen. Hematoxylin and eosin stain. × 97.

FIG. 3. Rat 21C, control, 5 days after experimental production of incised wound in back. Complete healing has occurred. Hematoxylin and eosin stain. × 97.

FIG. 4. Rat 21E, cortisone treated, 5 days after production of incised wound in back. There is complete absence of healing of the incision in the dermis, as well as a practically complete absence of inflammatory infiltration. The epithelium has grown across the gap, and there are a few fibroblasts immediately below the basal layer of the regenerated epithelium. Hematoxylin and eosin stain. × 97.



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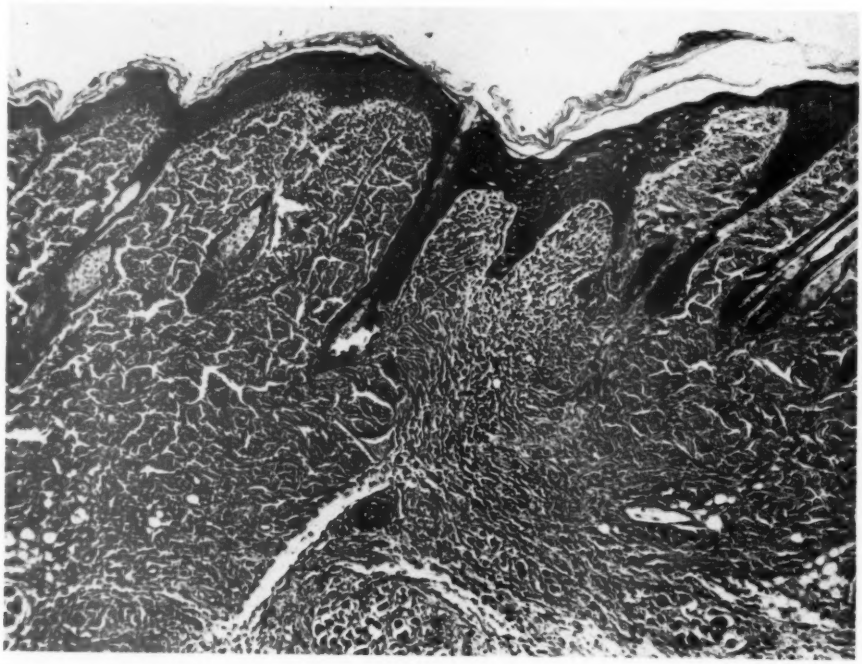

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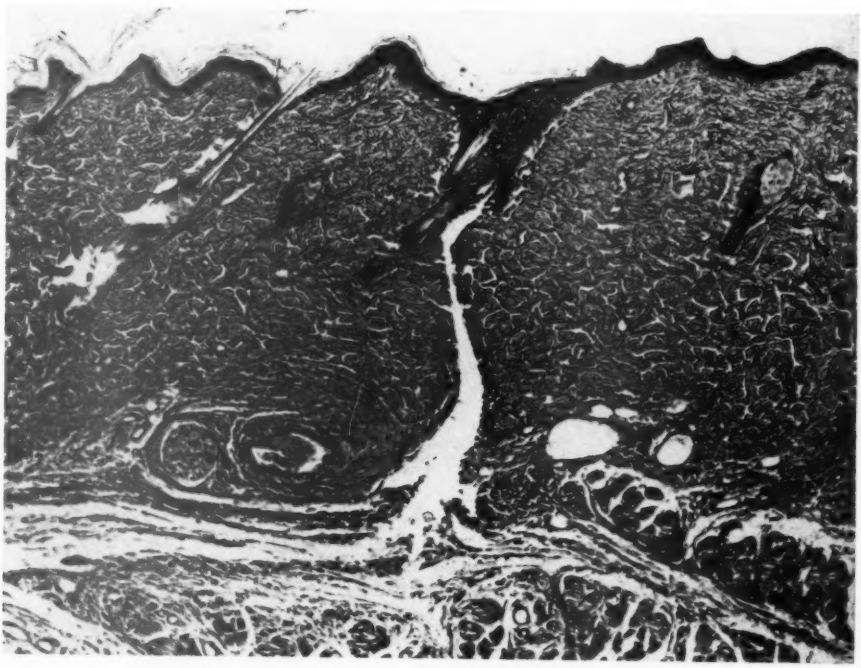

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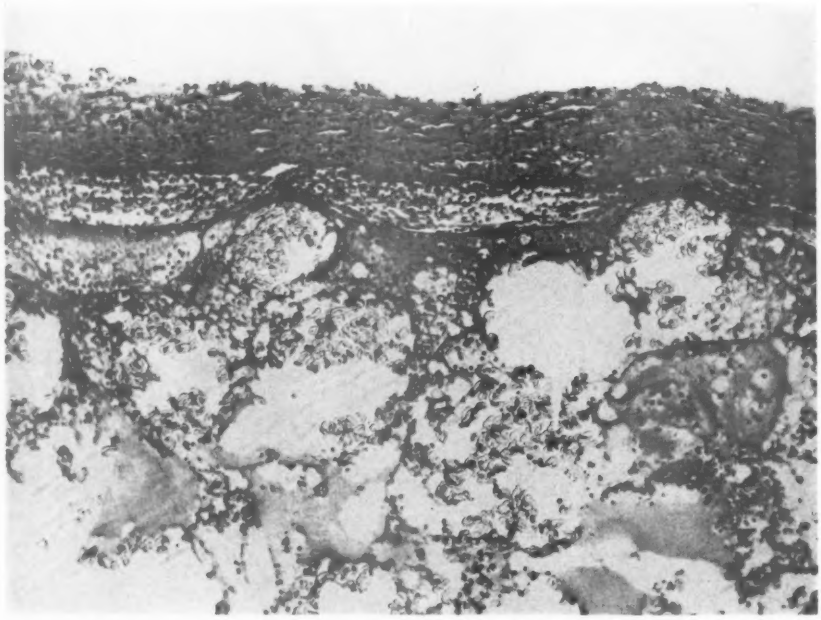
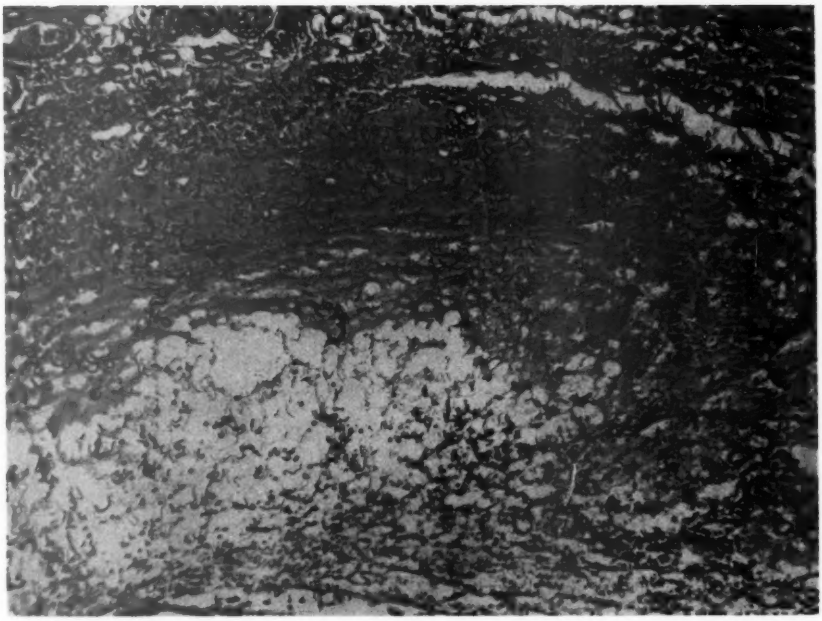
FIG. 5. Rat 13C, control, 24 hours after implantation of gauze pledget in subcutaneous tissue. Edema and leukocytic migration are evident. Above is a zone of compressed connective tissue. Hematoxylin and eosin stain. × 97.

FIG. 6. Rat 16E, cortisone treated, 24 hours after subcutaneous implantation of gauze pledget. Cotton fibers are not shown in this field. Edema and leukocytic migration are comparable with those of the control animal. Hematoxylin and eosin stain. × 97.

FIG. 7. Rat 21C, control, 5 days after subcutaneous implantation of gauze pledget. Fibroblastic and angioblastic proliferation are marked and there are numerous multinucleated giant cells of foreign body type associated with the cotton fibers. Hematoxylin and eosin stain. × 97.





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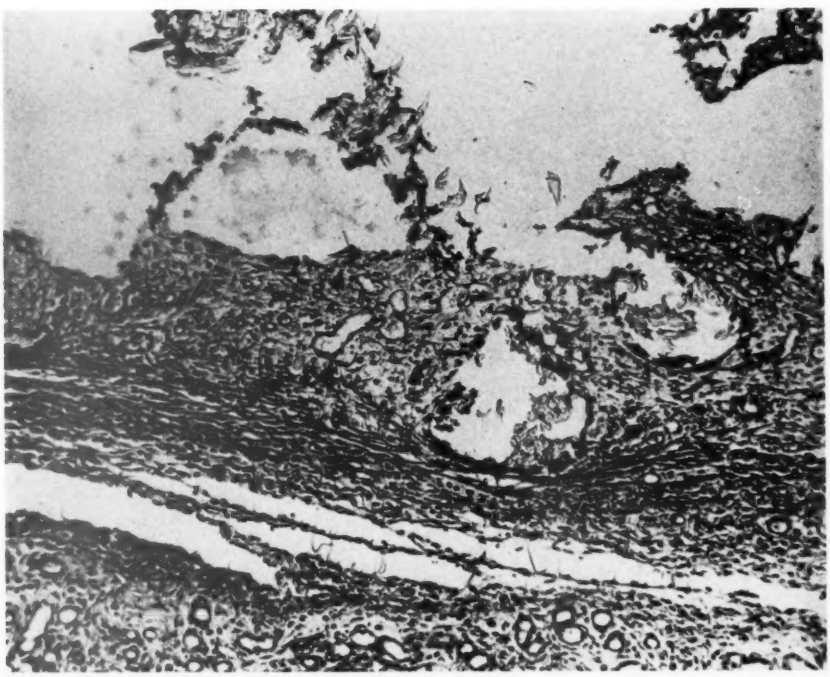

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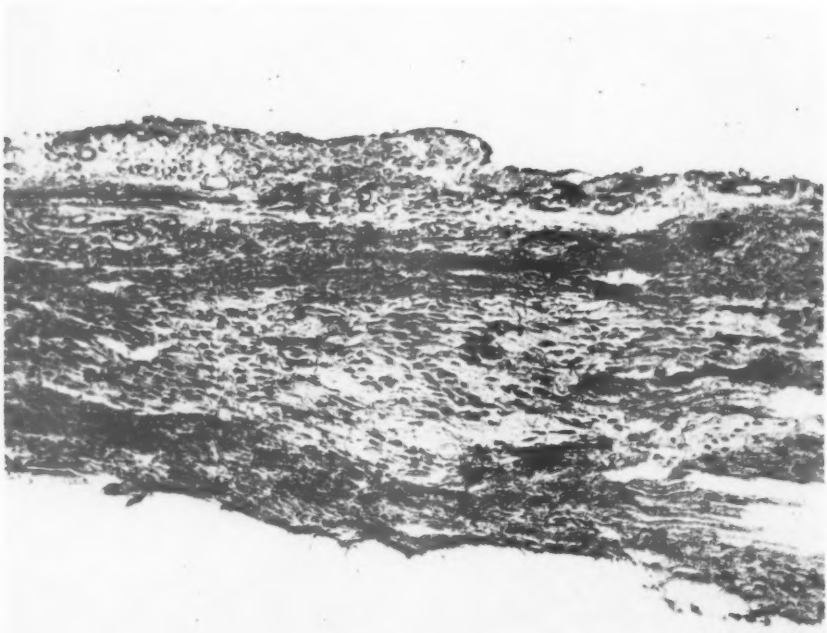
FIG. 8. Rat 21E, cortisone treated, 5 days after implantation of gauze pledget in subcutaneous tissue. There is no foreign body reaction and no blood vessel proliferation. The tissue represented in this photomicrograph was in immediate contact with the implanted foreign body, and consists of compressed collagenous fibers, some atypical fibroblasts, and mononuclear cells. Hematoxylin and eosin stain. × 97.

FIG. 9. Rabbit 35727-C, control, 2 hours after intradermic injection of *Bacillus coli* filtrate. Marked edema of the dermis, margination and migration of leukocytes through the wall of a dilated blood vessel may be seen. Hematoxylin and eosin stain. × 210.

FIG. 10. Rabbit 35729-E, cortisone treated, 2 hours after intradermic injection of *B. coli* filtrate. Edema, margination, and migration of leukocytes are present to a marked degree. Hematoxylin and eosin stain. × 210.



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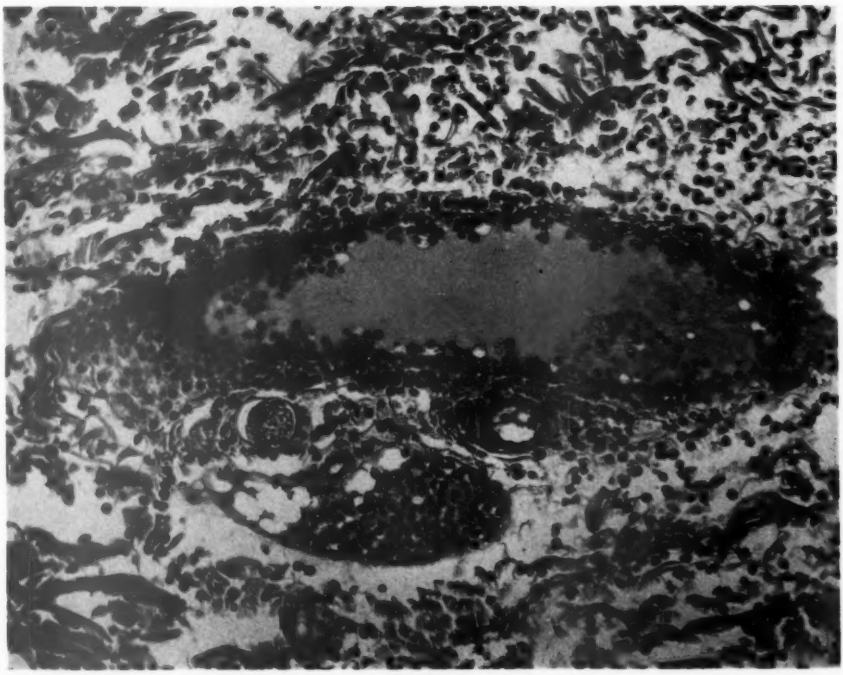

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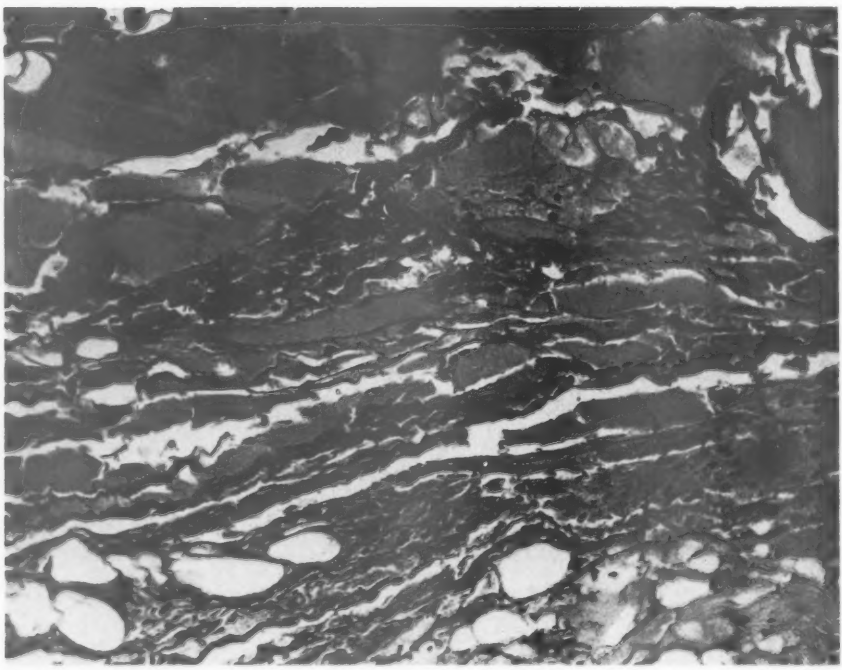
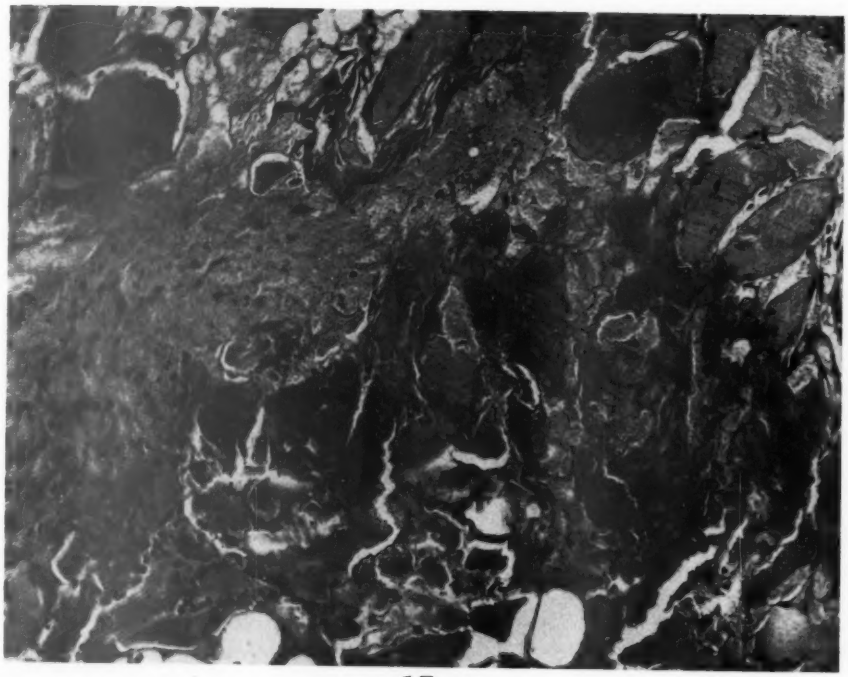
FIG. 11. Rabbit 124, control, 1 day following fracture of the femur. Section is from soft tissues immediately surrounding the fractured bone fragments. Of note are fragmentation and degeneration of striated muscle fibers, edema and hemorrhage, and a few polymorphonuclear leukocytes. Hematoxylin and eosin stain. × 210.

FIG. 12. Rabbit 119, cortisone treated, 1 day following fracture of the femur. Section is from an area comparable to that of Figure 11. Histologic findings are also similar to those of the control animal. Hematoxylin and eosin stain. × 210.

FIG. 13. Rabbit 127, control, 4 days following fracture of the femur. Fibroblastic proliferation and clusters of histiocytes in the empty sarcolemma tubes are shown. Hematoxylin and eosin stain. × 210.





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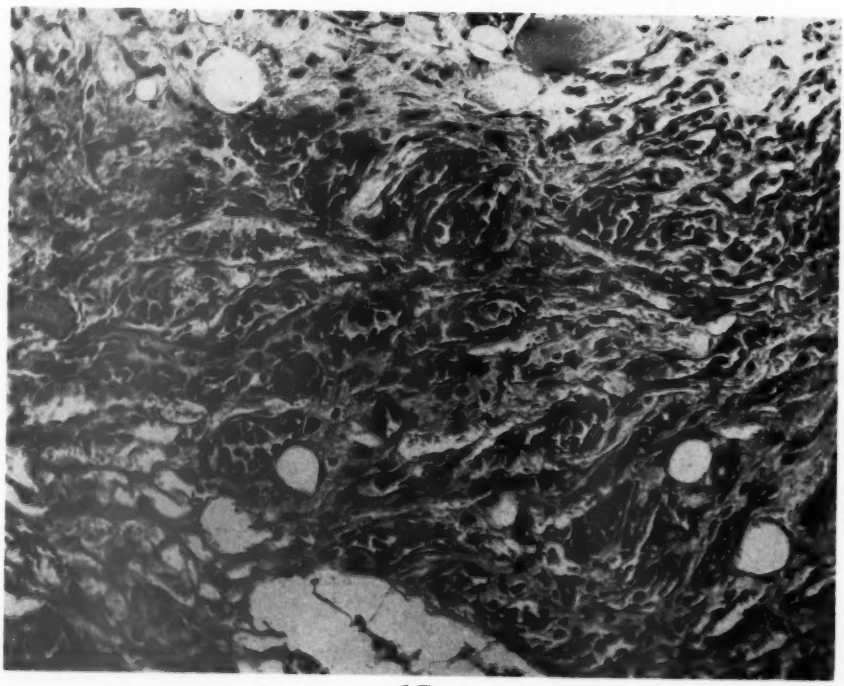

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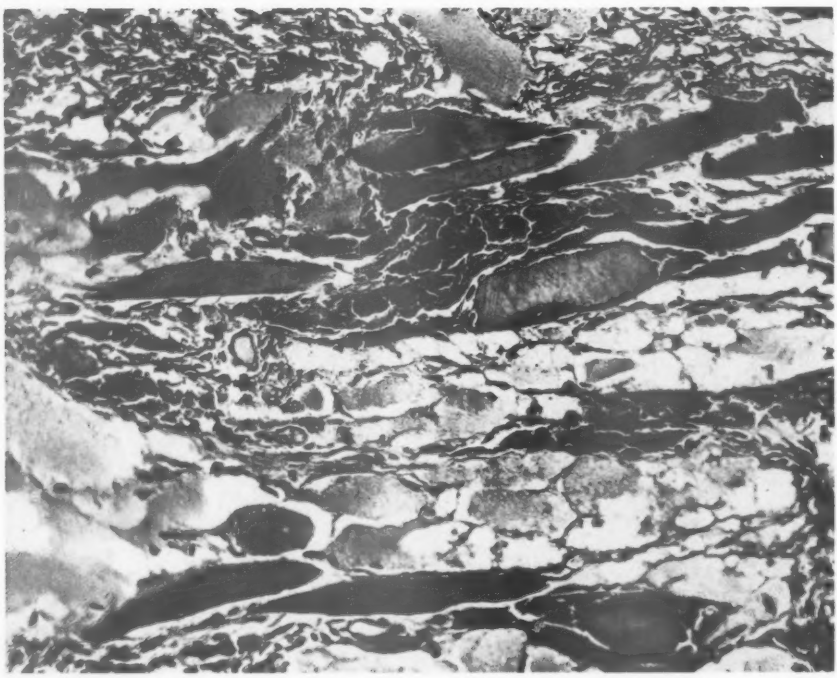
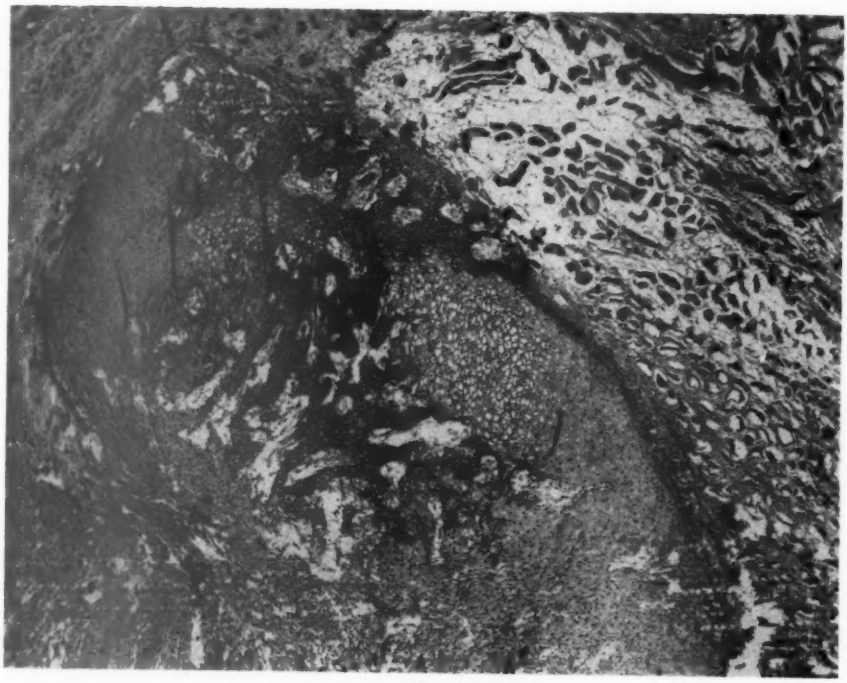
FIG. 14. Rabbit 120, cortisone treated, 4 days following fracture of the femur. There is a paucity of fibroblasts and of histiocytes in association with degenerated muscle fibers. Hematoxylin and eosin stain. × 210.

FIG. 15. Rabbit 169, control, 8 days following fracture of the femur. The osteoid and cartilaginous callus is well differentiated and there is moderate edema of the surrounding muscle. Hematoxylin and eosin stain. × 90.

FIG. 16. Rabbit 177, cortisone treated, 8 days following fracture of the femur. Of several sections taken, this was the only area showing reparative tissue with a pattern resembling atypical fibrocartilage. There is almost complete absence of blood vessels. Hematoxylin and eosin stain. × 210.





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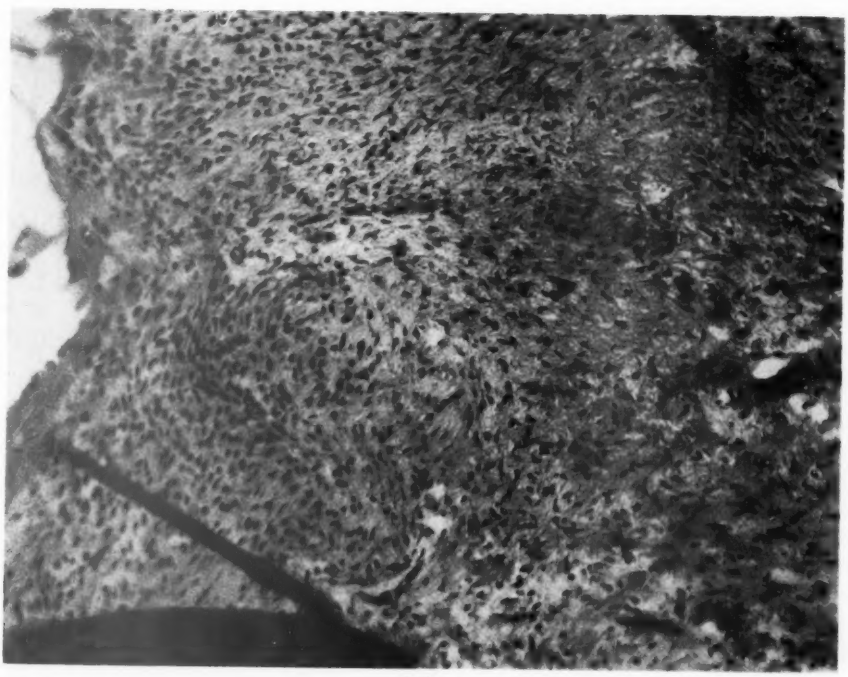

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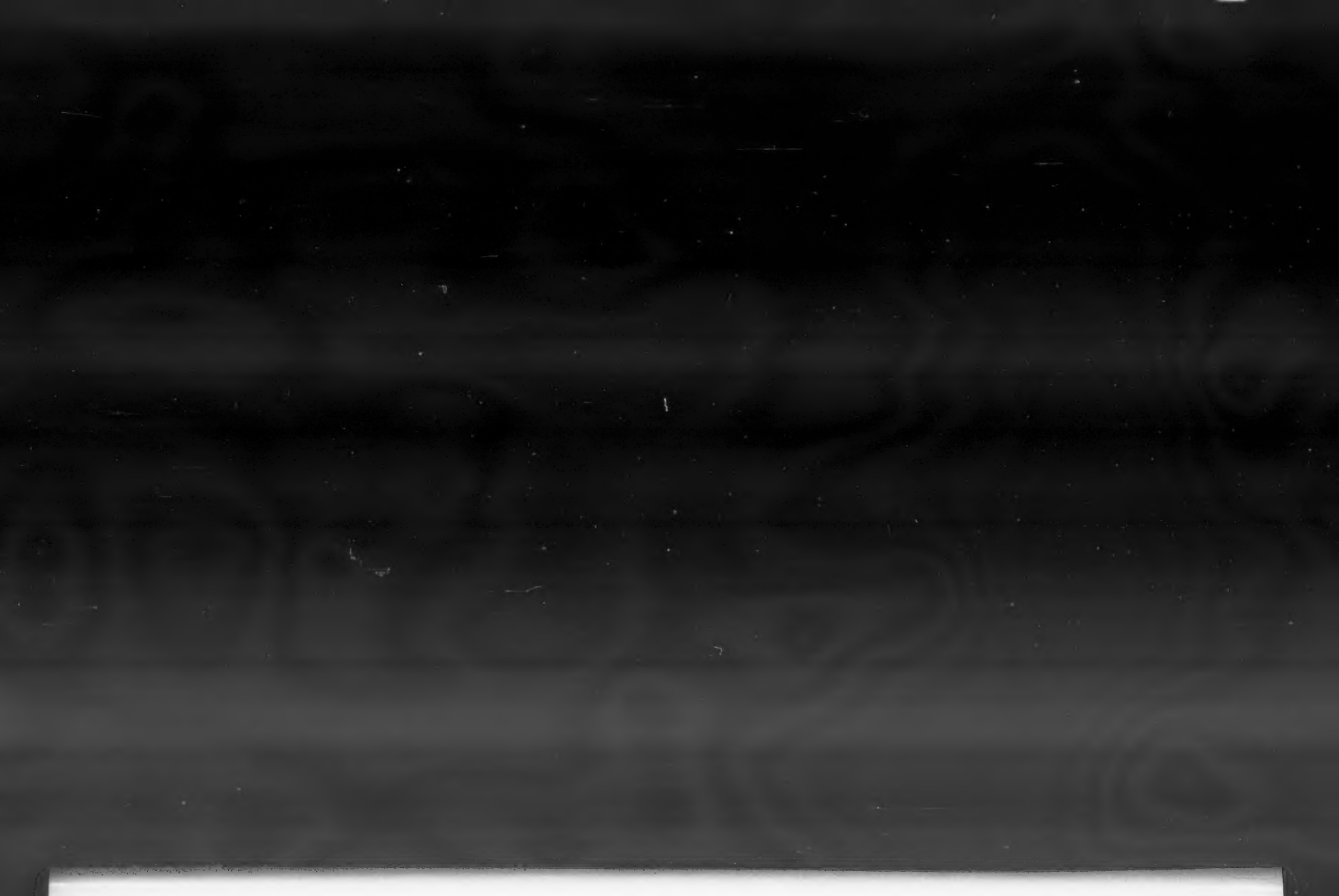
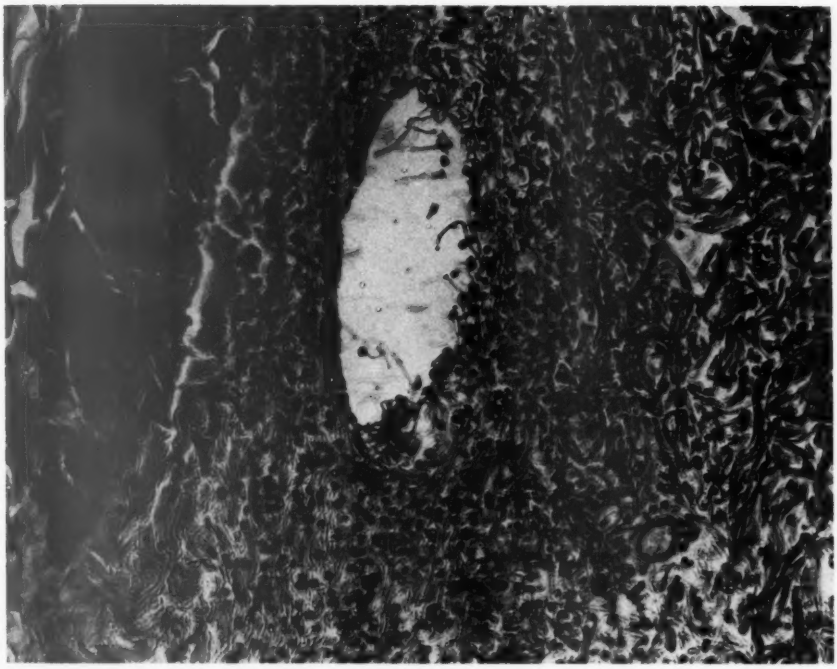
FIG. 17. Rabbit 694-C, control, 6 hours after intradermic injection of vaccinia virus. Edema, and infiltration with polymorphonuclear leukocytes may be seen. Hematoxylin and eosin stain. × 210.

FIG. 18. Rabbit 700-E, cortisone treated, 6 hours after intradermic injection of vaccinia virus. There is marked edema of the dermis, and infiltration with polymorphonuclear leukocytes. Hematoxylin and eosin stain. × 210.





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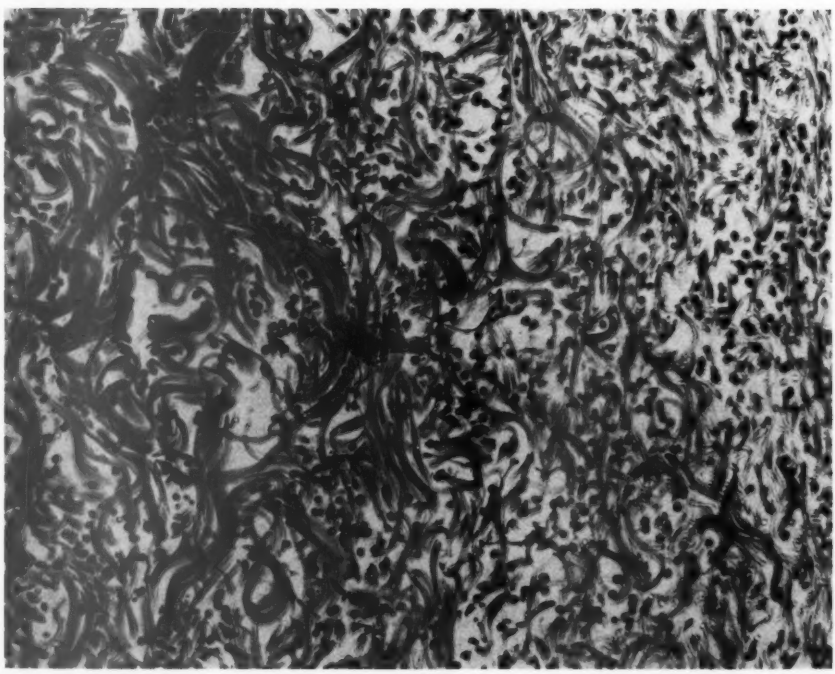

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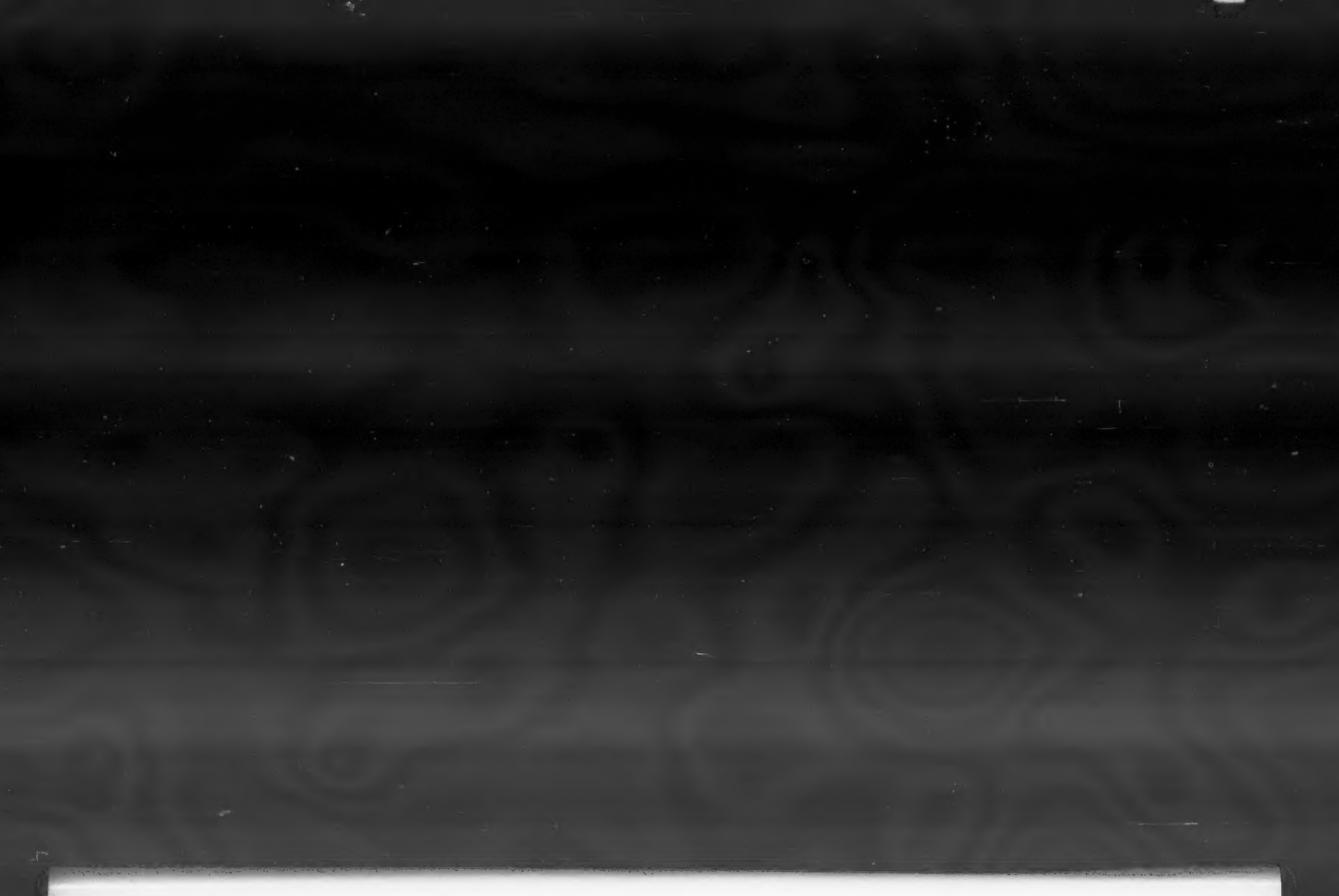
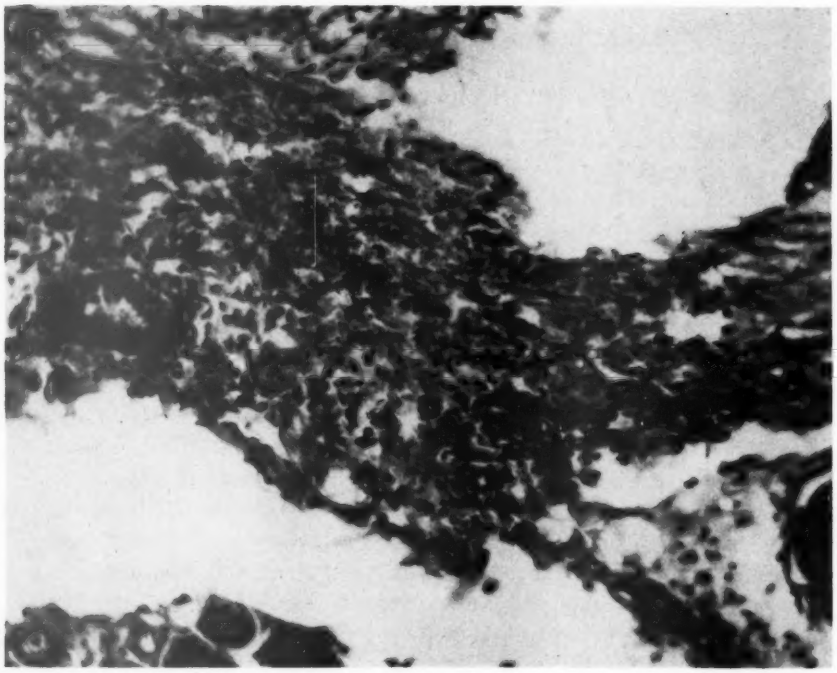
FIG. 19. Rabbit 699-C, control, 24 hours after intradermic injection of vaccinia virus. There are numerous partly degenerated polymorphonuclear leukocytes, and partly fragmented collagenous bundles. Hematoxylin and eosin stain. × 210.

FIG. 20. Rabbit 704-E, cortisone treated, 24 hours after intradermic injection of vaccinia virus. The only recognizable inflammatory cells are in a dry mass of exudate superficial to the partly destroyed epidermis. Hematoxylin and eosin stain. × 210.





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THE NORMAL GLOMERULUS AND ITS BASIC REACTIONS IN DISEASE *

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In this report it is our desire to set forth in some detail the finer structural features of the normal glomerulus revealed by utilizing new techniques and to point out the basic alterations which characterize its major diseases.

The fine structure of the glomerulus has long been the subject of study. Recent advances in histologic technique have contributed much and offer further promise in elucidation of the intimate detail of this important functional unit. Thus the periodic acid-Schiff technique sharply delineates the basement membrane.[1] Colloidal iron preparations counterstained with cochineal and picrofuchsin, as previously described by us,[2] clearly differentiate the epithelial and endothelial elements. The cytoplasmic product of the epithelial cells, in common with acid mucopolysaccharides, has a strong affinity for colloidal iron, staining blue, while the cochineal stains the endothelial cytoplasm orange-yellow. Collagen assumes the red coloration of fuchsin. A combined iron and periodic acid-Schiff stain has been reported by Ritter and Oleson[3] as well as by us.[2] We believe that the method described by us delineates acid mucopolysaccharides somewhat more sharply and selectively, due chiefly to the higher acidity of the colloidal iron used. Finally, important structural detail has been revealed by electron microscopy.

This study is based upon a series of special histologic techniques including those noted. A consideration of certain pathologic alterations has also served to clarify structural detail and relationships.

THE GLOMERULUS

There is general agreement that the glomerular loops are composed of endothelial cells and a basement membrane which is surfaced by epithelial cells. The exact nature of the basement membrane has not been established. Our studies would indicate that the basement membrane of the glomerulus has both endothelial and epithelial compo-

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nents which are most intimately related. The major component of the basement membrane appears to be a differentiated cytoplasmic product of the endothelial cells which stains with cochineal and is Schiff positive. This membrane is surfaced by epithelial cells which elaborate a substance of a mucoid character (mucoprotein or mucopolysaccharide) which completely covers the delicate hyaline endothelial membrane. This relationship is shown in Figures 1 and 3. The Schiff-positive endothelial membrane stains reddish violet and appears to be a continuous structure which is quite delicate at the tips and lateral margins of the loops. The surfacing layer of mucoid material, which stains blue, obscures the more delicate peripheral segments of the endothelial basement membrane, but with critical illumination and focusing it can usually be seen. It is accentuated in certain pathologic states. The epithelial cells and their mucoid cytoplasmic secretion occupy the intercapillary spaces and cover the outer aspects of the loops as well. Collagen is not seen in the normal glomerulus and rarely in pathologic conditions. Of special interest and of potentially great functional significance is the finding that the endothelial membrane is crossed by delicate extensions of the mucoid substance derived from the epithelium. In fortunate preparations delicate strands of mucoid can be seen to traverse the basement membrane and minute amounts of the mucoid material may be seen on the luminal side of the membrane (Figs. 1 and 3). It would appear that there are in effect minute canaliculi in the basement membranes which are occupied by the mucoid material. These may represent specialized channels for selective filtration.

The fine details of this remarkable structural relationship are most clearly shown by electron micrographs. Reports on glomerular structure utilizing electron microscopy have been made by Pease and Baker[4] and by Dalton.[5] For preparation of electron micrographs the technical methods which we have used are essentially the same as those reported by Dalton. Our interpretations of structural relationships in the electron micrographs have been made in light of observations utilizing other techniques in both normal and pathologic material. Kidney tissue from the rat was prepared by perfusion with osmic acid. This evidently has affinity for the mucoid element of the epithelium and probably preserves it in the state existing during life. So fixed, the cytoplasmic secretion of the epithelial cells is rendered moderately opaque to the electron beam. Frond-like extensions of the mucoid material show multiple attachments to the basement membrane of the glomerular loop. There is, in fact, but little "space" between glomeru-

lar loops which is not occupied by the mucoid cytoplasm of the epithelial cells and its extensions. Pease and Baker considered the tongue-like projections to be integral parts of the basement membrane itself. Dalton, on the other hand, considered the ridges to be cytoplasmic processes of the epithelial cells applied to the basement membrane. On this point, our observations are in agreement with Dalton. Neither Pease and Baker nor Dalton relate the basement membrane proper to a cell. In fact, Dalton interprets his electron micrographs as showing a very delicate endothelial membrane internal to the basement membrane proper. This concept is difficult to reconcile with histochemical preparations which seem to indicate that the cochineal staining or Schiff-positive cytoplasm surrounding endothelial nuclei is the same as the finer extensions of the membrane. From the study of electron micrographs as well as histochemical preparations it appears that delicate cytoplasmic projections of the epithelial cells in fact cross the basement membrane. Figure 11 shows tongue-like processes of epithelial cells attached at many points to the endothelial membrane. From these points a mucoid substance appears to be thinly spread over the membrane and in fact to traverse this membrane at regular intervals in a manner analogous to the cement lines separating hexagonal tile. This is shown in Figure 11 and in the semi-diagrammatic representation in Figure 12.

Pathologic Changes in Glomeruli

The histochemical staining techniques employed in this study serve to delineate changes in glomerular structure somewhat better than older methods. Furthermore, study of abnormal glomeruli with these methods has aided interpretation of normal glomerular structure and of the histogenesis of the lesions.

In glomerulonephritis the pathologic changes are primarily of a proliferative character, involving both endothelial and epithelial cellular elements. Proliferation of endothelial cells is the major feature in most cases of glomerulonephritis. With endothelial cell proliferation there is reduplication of the endothelial cytoplasmic element of the glomerular loop. With the colloidal iron-cochineal technique this material stains yellow-orange (Fig. 4) and in the combined colloidal iron-Schiff technique it stains a crisp red (Fig. 2), which are the normal staining properties of the endothelial element of the capillary loop. The material is not collagenous. This is best seen in preparations stained with the colloidal iron and cochineal technique in which the basement membrane stains an orange-yellow, contrasting with the red-

staining collagen (Figs. 8 and 9). While the Schiff reaction stains the endothelial element of the basement membrane sharply, it does not differentiate it clearly from collagen. If the surface epithelium of the glomerular loop proliferates, it forms a variable amount of mucoid substance, but apparently not as much as the normal epithelial cell (Fig. 2). Proliferation of the endothelium in the glomerular loop cuts down the lumen and the normal endothelial-epithelial cell arrangement is distorted. Naturally both factors seriously impair the functional capacity of the glomerulus.

The glomerular lesion of disseminated lupus in its most characteristic form shows a striking hyaline thickening of the basement membrane, chiefly the endothelial element (Fig. 7). In addition the staining property of this membrane is somewhat altered. It stains rather brilliantly with eosin, and with the Schiff reaction the color may be in part orange rather than the simple red color. It seems likely that this color change is related to suffusion of the loop with some element of the plasma proteins, possibly fibrinogen. There may be also some granularity and swelling of the mucoid material of the epithelium. The appearances suggest that the material may be depolymerized and washed away with the glomerular filtrate. Casts with a blue mucoid staining reaction are commonly seen in tubules (Fig. 7). The basic pathologic reaction of lupus appears to involve a swelling of the connective tissue ground substance which becomes suffused with plasma protein,[6] and the deposition of plasma proteins including fibrin in the mucoid matrix is probably the basis of the characteristic "fibrinoid" degeneration. Not infrequently in lupus there is also a degree of proliferative change in the glomerular endothelium which is not fundamentally different from that occurring in proliferative glomerulonephritis.

In uncomplicated essential hypertension there is relatively little alteration of the glomeruli. They may show variable thickening of the endothelial element of the basement membrane. In the more severe cases this thickening may be considerable, and in the malignant form of essential hypertension the sclerotic arterioles also show necrosis with thrombosis causing varied degrees of injury to the glomeruli (Fig. 6).

The glomerular lesions of eclampsia bear some resemblance to the so-called membranous glomerulonephritis encountered in the nephrotic syndrome.[7] The endothelial element of the eclamptic glomerulus appears thickened and may show some longitudinal fibrillation. Most glomeruli are essentially devoid of blood (Fig. 5). In several cases

which we have had the opportunity to examine there were also alterations that are analogous to those of so-called lower nephron nephrosis, with partial disruption of the distal convoluted tubules and with the formation of granular hemoglobin-containing casts. There may be extensive accumulation of such hemoglobin casts in the loops of Henle and in the collecting tubules.

The glomerular alterations so frequently encountered in diabetes are of considerable interest and aid in the interpretation of the structural arrangement of the glomerulus. There is a striking thickening of the endothelial element of the basement membrane, particularly in the axial segments of the glomerular loops. The endothelial layer can usually be very clearly outlined in its entirety, which because of its delicacy is often difficult to demonstrate in the normal glomerulus. In the axial portions of the loops a broad layer of endothelial cytoplasm surrounds the nuclei, and as the cytoplasm extends away from the nuclei the endothelial layer becomes less prominent, but it can be outlined in its entirety including the thin segments of the peripheral loops. This endothelial cytoplasm is orange-yellow in preparations stained by colloidal iron and counterstained with cochineal. It is clear that this material is not collagen. The designation of "intercapillary glomerulosclerosis" appears to be a misnomer. In the first place collagen is not a component of the lesion and, in the second place, the lesion is, in our opinion, not in the "intercapillary" space but actually represents a basement membrane alteration.

Summary

Structural detail of the normal glomerulus has been studied by new histochemical techniques and electron micrographs. The basement membrane is considered to be primarily a differentiated cytoplasmic product of the endothelial cells. The surfacing epithelial cells elaborate a cytoplasmic secretion with properties of a mucopolysaccharide or mucoprotein. This substance is attached at many points to the basement membrane and is thinly spread over its entire surface. Evidence derived chiefly from electron micrographs is presented which indicates that the epithelial mucoid penetrates the endothelial basement membrane at regular intervals of approximately 0.12 μ.

Changes which are considered to be the basic alterations in structure occurring in the major glomerular diseases are presented briefly and illustrated.

We are much indebted to Mr. Ernest A. Naumann for the preparation of the colored photomicrographs.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Distal segment of an essentially normal glomerulus stained by the combined colloidal iron and Schiff technique. The axial segments of the basement membrane stand out in red. The peripheral segments of the loops also stain red but cannot be seen clearly in this photograph. The deep blue-stained cytoplasm of the epithelial cells surfaces this Schiff-positive membrane. Careful inspection shows minute strands of bluish, presumably mucoid, material traversing the basement layer. A few red blood cells staining orange may be seen in the lumina of the capillary loops. × 600.

FIG. 2. Two glomeruli showing the changes of proliferative glomerulonephritis. Endothelial cell proliferation is evident with new formation of the Schiff-positive (red) endothelial cell cytoplasm or basement membrane. At the upper pole of the glomerulus shown above there is an epithelial crescent. It will be noted that the cytoplasmic extensions from these cells stain with colloidal iron. Colloidal iron-Schiff technique. × 130.

FIG. 3. Hilar segment of an essentially normal glomerulus stained by colloidal iron and cochineal. The primary element of the basement membrane here stains yellow. It will be noted that this yellow cytoplasm surrounds endothelial nuclei, indicating that this is a cytoplasmic product of the endothelial cell. A thin layer of similarly staining yellowish cytoplasm may be seen at the periphery of the capillary loops. This is not clearly shown here (see Fig. 1). The deep blue cytoplasm of the epithelial cells surfaces all of the glomerular loops. A few red blood cells are seen in the efferent arteriole. × 600.

FIG. 4. Glomerulus of proliferative glomerulonephritis stained by the colloidal iron-cochineal technique. The cells in excessive numbers are endothelial, and it will be seen that the surrounding cytoplasm stains yellow as does the normal basement membrane. The epithelial elements appear crowded out. A few epithelial cells surrounded by blue cytoplasm are seen, and some such bluish cytoplasm covers portions of the original outer aspects of glomerular loops. × 390.

(Legends continued on following page)

FIG. 5. Glomerulus and adjoining tubules from a case of eclampsia, stained by the colloidal iron-cochineal technique. The endothelial basement membrane is seen to be thickened and is clearly visible at the periphery of the glomerular loops as well as in the axial portions. The glomerulus is relatively avascular. The epithelial cells are inconspicuous, and their cytoplasm appears to be reduced and shows some granular dispersion. The adjoining distal convoluted tubules show degeneration of the lining cells and contain a granular hemoglobiniferous material analogous to that seen in so-called lower nephron nephrosis. × 300.

FIG. 6. Glomerulus and arteriole from a case of malignant hypertension. There is smudgy necrosis of a portion of the arteriolar wall as well as of some of the Schiff-positive endothelial cytoplasm at the hilus of the glomerulus. Epithelial cells are conspicuous, and some have desquamated. Colloidal iron-Schiff technique. × 130.

FIG. 7. Glomerulus and adjoining tubules from a case of acute disseminated lupus. The glomerular loops are bright red and clearly thickened. A few epithelial cells with blue cytoplasm are evident at the surfaces of the loops. Casts staining blue with colloidal iron are present in adjoining distal convoluted tubules. Colloidal iron-Schiff technique. × 65.

FIG. 8. Glomerulus from a case of so-called diabetic "glomerulosclerosis," stained by the colloidal iron-cochineal technique. It is evident that there is a marked thickening of the axial segments of the glomerular basement membranes. Endothelial cell nuclei are clearly seen, surrounded by an orange-yellow cytoplasm. The blue-staining epithelial cytoplasm can be seen surfacing glomerular loops and occupying the intercapillary spaces. The red-staining substance lying between some of the adjoining tubules is collagen. A zone of thickening of the parietal basement membrane is visible in the lower right-hand portion of the glomerular capsule. × 130.

FIG. 9. Detail of a segment of the glomerulus shown in Figure 8. Here the endothelial nuclei are seen to be surrounded by orange-yellow cytoplasm. There is pronounced hilar thickening of the axial segments of the endothelial basement membrane. The delicate but, in fact, thickened peripheral portions of this basement membrane may be seen. Some of the thickened endothelial membrane appears to be traversed by delicate strands of bluish material, presumably the cytoplasmic product of the epithelial cells. The capsular basement membrane, seen above, stains, as does that in the glomerular loops. The red-staining substance adjoining is collagen. × 390.

FIG. 10. Another glomerulus from a case of diabetic "glomerulosclerosis," with the formation of a distinctive, hyaline, ball-like structure. This is evidently a swelling or excess deposition of the endothelial cytoplasm. The blue-staining substance lying between the tubules is the connective tissue ground substance. The red-staining material shown in the lower left of the photograph is collagen. The absence of collagen in the glomerulus may be noted. × 130.

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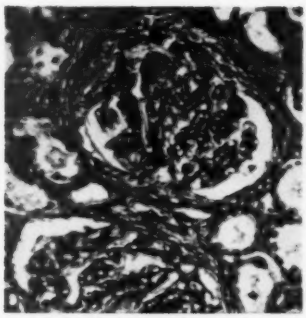

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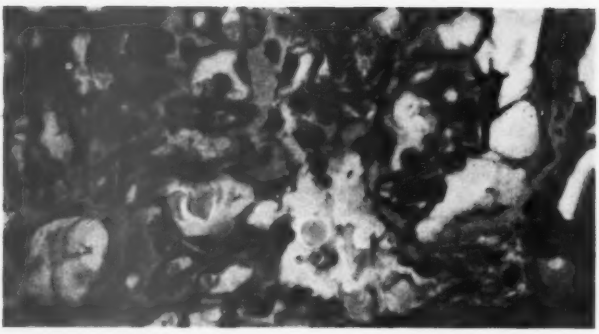

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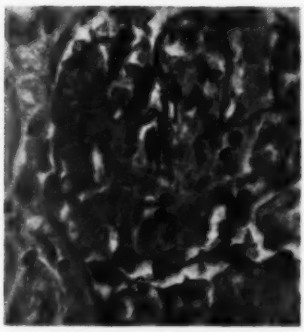

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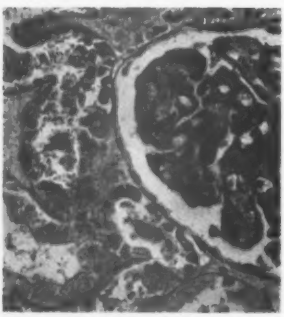

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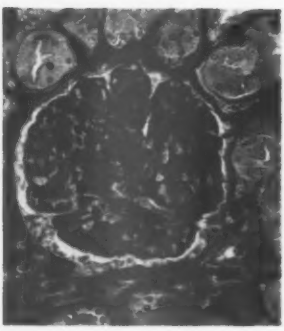

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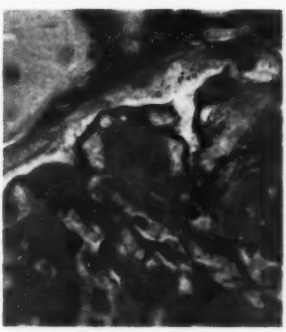

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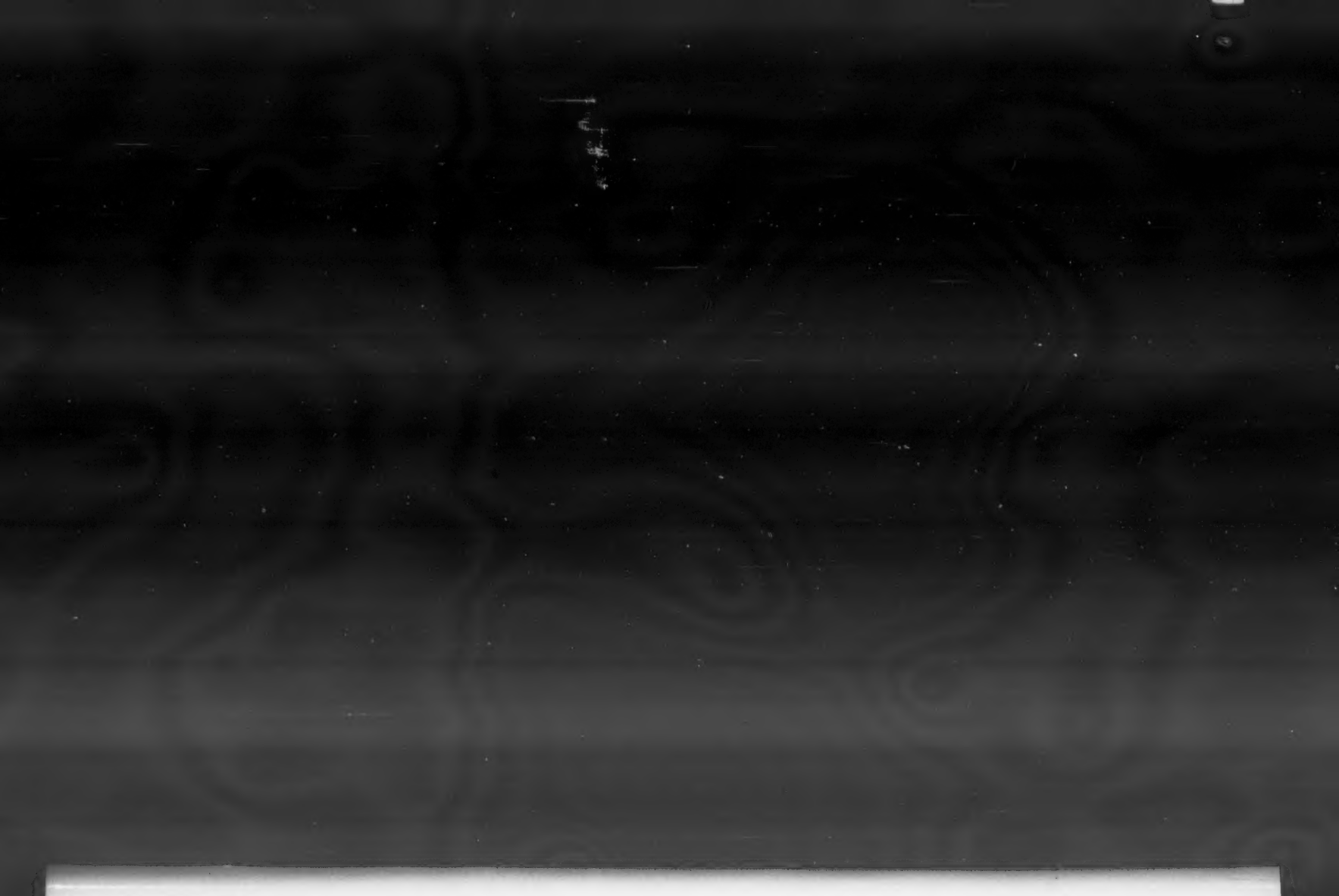
FIGS. 11 and 12. An electron micrograph of a segment of rat glomerulus perfused with osmic acid (Fig. 11). Below (Fig. 12) is a semi-diagrammatic representation of the same field. Endothelial cell nuclei (EN) lie inside the capillary loops. Cytoplasm of the endothelial cell is believed to form the primary layer of the basement membrane. An epithelial cell (EP) is shown at the lower right. The cytoplasm of such cells gives rise to frond-like extensions of mucoid character (MC) which are applied to the surface of the endothelial membrane. This substance appears to penetrate the endothelial membrane at regular intervals (area A). A tangential section (T) shows a periodic penetration of the endothelial membrane by the epithelial mucoid cytoplasm, resembling cement lines in tile. × 5900.



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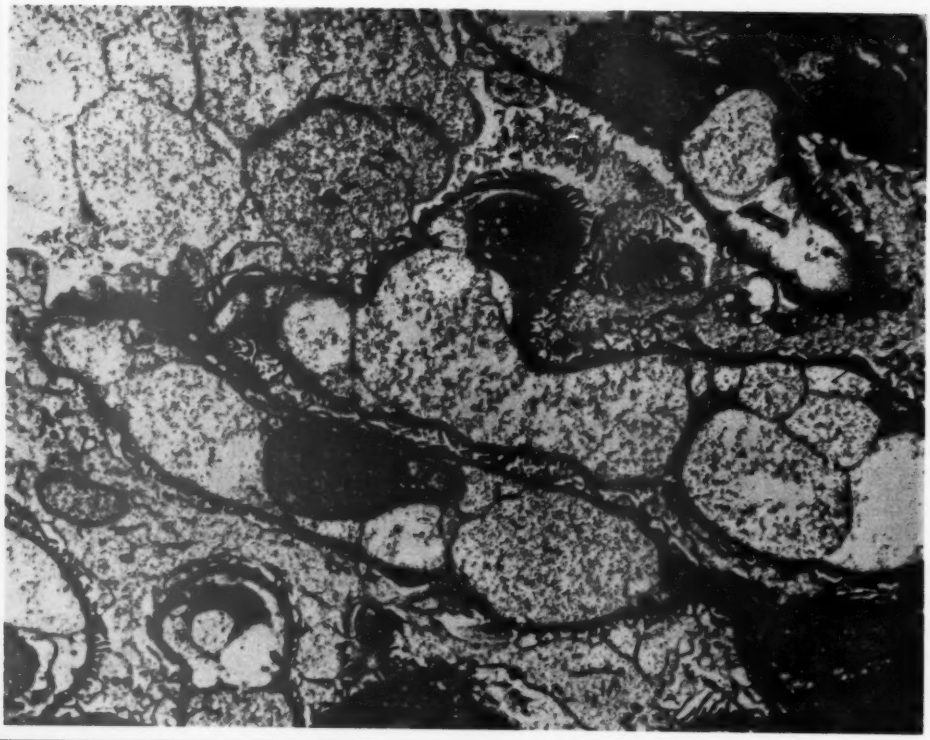

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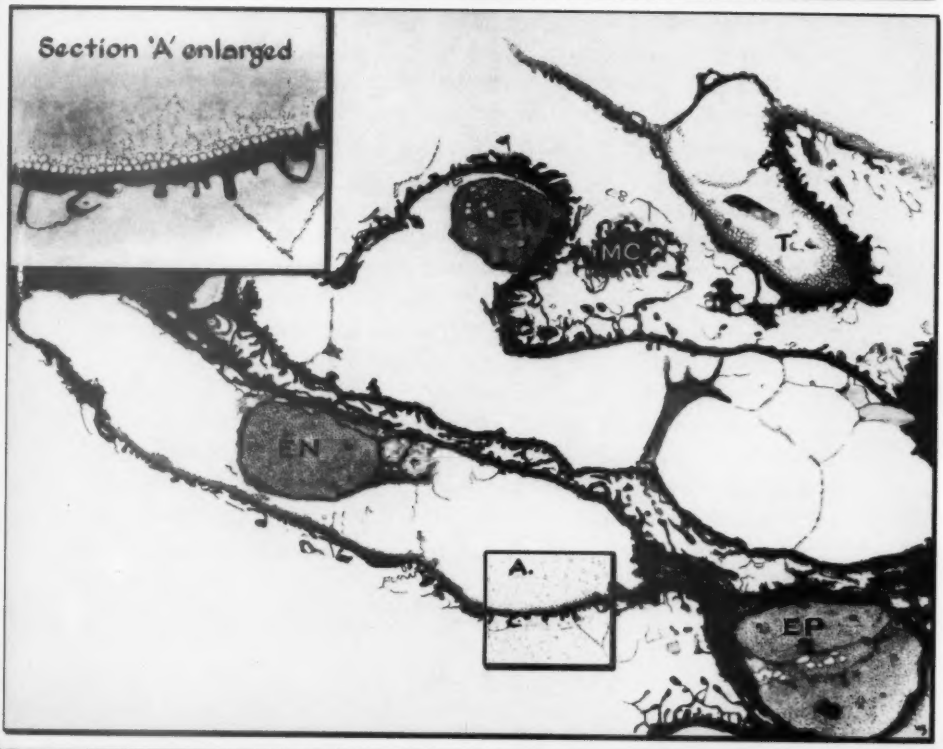

GLOMERULONEPHRITIS *

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Glomerulonephritis is essentially inflammation and repair of glomeruli, but various interpretations of the morphologic changes in the disease process have been complex and confusing. It is believed that an orderly, simple process completely in line with inflammation of other organs must be present. In a previous report an outline of these changes was presented.[1] The present paper is an attempt to clarify further the pathologic changes in the glomerulus in the various stages of diffuse glomerulonephritis.

Throughout the body a fundamental capillary-connective tissue-epithelium structural relationship is maintained. Where epithelial cells lie against connective tissue, a basement membrane which is a connective tissue product and apparently dependent on fibroblasts for its integrity is present. All capillaries have their own delicate basement membrane. Between capillary basement membrane and epithelial basement membrane, varying amounts of connective tissue substance (ground substance, collagen, elastin) are present.[2] It is inconceivable that this fundamental relationship should not be maintained in the glomerulus as it is in the rest of the kidney and other organs. That the glomerulus has such a structure has been recognized by Zimmermann,[3] MacCallum,[4] Jones,[1] Kimmelstiel,[5] Oberling,[6] and Goormaghtigh.[7] The normal glomerulus, therefore, consists of complex loops of capillaries having their own delicate basement membrane. An epithelial basement membrane and the covering glomerular epithelium extend around Bowman's capsule and are reflected over the capillary loops as the peritoneum is reflected over the small bowel. Thus between an endothelial and epithelial cell are an endothelial basement membrane, a pericapillary connective tissue space, and an epithelial basement membrane. In the places between capillaries, opposite layers of epithelial basement membrane almost meet, but leave a small space (the so-called intercapillary space,[8] the glomerular stalk,[1] the mesangium[3]). In the normal glomerulus the interstitial space of the stalk is visible, while the pericapillary interstitial space is usually too thin to be visualized. In secondary amyloidosis the amyloid may fill both these spaces, thus demonstrating their presence microscopically. Similarly in eclampsia, glomerular edema results in a filling of these spaces with edema fluid, permitting one to see clearly both epithelial and endothelial basement

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membranes. In the interstitial space of the stalk lie cells of fibroblastic origin (Figs. 1 and 2). In childhood these cells are somewhat less numerous than either epithelial or endothelial cells, but by 30 years of age their number has increased to approximately that of endothelial and of epithelial cells.[1] Study of serial sections cut at 1 to 2 μ and stained with periodic acid-Schiff's technique or periodic acid-silver stain is a prerequisite for recognition of these finer details of the glomerulus.

It is believed that the time-honored concept of a glomerular tuft composed only of endothelium, capillary basement membrane, and epithelium without an interposed connective tissue space containing connective tissue cells is not only inconsistent with the general structural organization of the body, but cannot adequately explain the various changes which occur in diseases such as eclampsia,[1] amyloidosis,[1,4] and glomerulonephritis.[1,4]

Materials and Methods

For this study, 26 cases of acute diffuse glomerulonephritis, 3 of subacute glomerulonephritis, 4 of lipoid nephrosis, and 15 of chronic glomerulonephritis were selected for careful morphologic study. The cases of acute glomerulonephritis may be classified as follows: exudative, 10 cases; exudative and proliferative, 10; proliferative, 2; capsular proliferative, 3; and hemorrhagic, 1. The 3 subacute glomerulonephritis cases were of the subacute capsular variety. The 4 cases of lipoid nephrosis were all from children under 10 years of age. The cases of chronic glomerulonephritis were generally of the usual type except for 2 which were of the so-called chronic lobular[9] type.

Tissue fixed in 10 per cent formalin, Bouin's fluid, Carnoy's fluid, and Zenker's fluid was cut at 2 μ. Double embedding in 3 per cent celloidin and paraffin was used occasionally. Routine necropsy blocks may be readily cut at 2 μ if paraffin at the sides of a relatively rectangular block is trimmed off so that the embedded tissue comes to each lateral border and if both paraffin block and microtome knife are kept cold with ice.

Hematoxylin and eosin, McManus' periodic acid-Schiff's stain, periodic acid-Gomori's silver methenamine stain, Mallory's phosphotungstic acid hematoxylin, Foot's modification of Masson's trichrome stain, and Weigert's fibrin stain were used. The McManus stain and periodic acid-methenamine silver stains were the most useful. For clarity, the latter stain on sections cut at 2 μ is difficult to surpass.

For reasons unknown there are certain differences between the McManus periodic-Schiff's reaction and the periodic acid-Gomori's

methenamine silver reaction. With the silver stain, fibrin, fibrinoid and certain connective tissue hyalins are negative, while with the McManus stain they are positive. On the other hand, collagen fibers stain intensely with silver, while very weakly or not at all with McManus' stain. With Carnoy's fixation the staining of collagen by silver is somewhat depressed and therefore the basement membranes of capillaries are shown better than by any other method. With this silver technique any counterstains may be used. Double staining with hematoxylin and eosin is very useful as a counterstain, and Masson's trichrome is useful for its differential staining.

Acute Glomerulonephritis

Inasmuch as acute glomerulonephritis is an acute inflammatory reaction in the renal glomerulus, the various histologic findings must be interpreted in terms of the basic changes of inflammation as seen in all organs. Since the same basic anatomical relationships of endothelium, epithelium, basement membranes, and connective tissue space are present in the glomerulus as in other organs, there should be no basic difference between the various stages of inflammation in other organs and in the glomerulus.

Vascular Changes. The earliest phases of acute inflammation are those of physiologic vasoconstriction followed by prolonged vasodilation. Although these processes occur, without doubt, in the glomerulus, they are not amenable to specific pathologic recognition. Following vasodilatation increased capillary permeability results in the extravasation of inflammatory edema fluid through the capillary endothelium and basement membrane into the connective tissue spaces. In the glomerulus this is manifested by an accumulation of fluid in the potential connective tissue spaces of the stalk or mesangium, and to a lesser degree in the potential space between the capillary basement membrane and the epithelial basement membrane (pericapillary interstitial space). The edema fluid strips the capillary and epithelial basement membranes apart and, at the same time, balloons out the epithelial basement membrane. This results in irregular club-shaped lobules of the glomerulus with the epithelial basement membrane forming the peripheral outline of each clubbed lobule. The capillaries bounded by their basement membrane may float relatively free in edema fluid or they may still be attached in part to the epithelial basement membrane (Figs. 3 and 4). Strands of fine fibrillar material, staining like basement membrane substance, are seen to be frayed apart by this edema fluid. These strands insert in part into epithelial basement membrane and in part into capillary basement membrane. These fibrils are pre-

dominantly in the region that was originally the relatively tiny connective tissue space of the stalk or mesangium. This is the fibrillar connective tissue normally present in the glomerulus. A few delicate fibrils apparently connect epithelial and endothelial basement membranes about the capillaries. These frayed fibrils of normal glomerular connective tissue are the so-called intracapillary fibrils of Bell.[10] That some of the protein-rich edema fluid passes through the epithelial basement membrane and the epithelium into Bowman's capsule is readily recognized by the strands of fibrin, and clouds of precipitated protein seen in Bowman's space or in the tubules. In some cases of glomerulonephritis the diapedesis of red blood cells into the urine is prominent. Lobar pneumonia and other types of inflammation show similar diapedesis.

The Cellular Phase of Exudation. In the injured glomerulus the increased capillary permeability is associated, as in other examples of inflammation, with a so-called increased stickiness of the endothelial cells. Circulating polymorphonuclear neutrophils adhere to these sticky walls and thus accumulate in the glomerulus. This frequently occurs before much glomerular edema has formed. At this stage one sees a relatively normal glomerulus with many neutrophils in the capillary channels, which certainly corresponds to the phenomenon of *margination* of leukocytes seen in most acute inflammatory processes (Figs. 5 and 6). After adhering to the endothelial wall, the leukocytes migrate through the endothelial cell wall and endothelial basement membrane into the interstitial space of the connective tissue stalk and the pericapillary interstitial space (Figs. 7, 8, 9, and 10). Many of the neutrophils continue their migration through the epithelial basement membrane and glomerular epithelium and enter Bowman's capsule. It is in this manner that they may appear in the urine. Monocytic cells are present in small numbers even in early stages of most acute inflammatory reactions. As time passes, the relative proportion of these monocytic cells is greater. In the glomerulus the same feature is seen and the monocytic cell is sometimes the predominant inflammatory cell. It is believed that most of the so-called endothelial proliferation of proliferative glomerulonephritis is actually based upon a misinterpretation of the migration of histocytic cells into the interstitial spaces of the glomerulus.

In view of the tremendous variations that can occur in the appearance of histocytic cells under different environmental conditions, *i.e.*, Kupffer cells, epithelioid cells, microglia, Aschoff cells, it is not surprising that in the presence of acute glomerular inflammation it is

practically impossible to state with assurance that any individual cell is a fibroblastic cell or is a histiocytic cell. It is for this reason that in the illustrations of this manuscript differentiation is not always attempted.

In correlation with the clinical picture of these cases it was found that duration of the disease process did not explain entirely why in some cases polymorphonuclear neutrophils were the predominant exudative cell, and in others a mononucleated cell was more prominent. In 2 cases with blood cultures positive for *Staphylococcus aureus*, the most severe degree of neutrophilic glomerular exudation was seen. It is believed that, to a certain degree, the type of exudative cell which is predominant may be due to variations in the type of chemotaxis (varieties of allergic phenomena) and severity of injury.

The Cellular Reaction to Injury. Endothelial, epithelial, and connective tissue cells of the mesangium or glomerular stalk all show cytoplasmic swelling, nuclear enlargement, and increased nuclear vesiculation. This response is commonly seen in inflammation elsewhere, although necrobiotic changes are rarely seen.

As a result of the injury initiating diffuse acute glomerulonephritis, the connective tissue substance sometimes suffers a peculiar focal injury so that an eosinophilic hyaline material distends the interstitial space of a portion of an occasional glomerulus (Fig. 11). This results in a "wire-loop" lesion like that seen in acute disseminated lupus erythematosis.[9] The basement membranes, both epithelial and endothelial, which bound the fibrinoid substance, are attenuated and may practically disappear for short distances in areas of most severe involvement. Embedded in this fibrinoid material is a sparse, extremely delicate network of connective tissue fibrils. This network is seen only after the periodic acid-silver stain with which the fibrils are black against the background of fibrinoid substance stained by whatever counterstain is used.

The fibrin thrombi which form in capillaries as a result of severe endothelial injury can be confused with this fibrinoid substance. As these may occur along with fibrinoid in the same glomerulus, differentiation may be difficult. Fibrin thrombi may be recognized by lying relatively free within the lumina of the capillaries, by the morphologic structure of the fibrin threads forming the thrombus, and by the absence of connective fibrils within the material.

The Effect on the Glomerular Circulation. That the glomerulus of acute glomerulonephritis is relatively free of red blood cells is well known. On an anatomical basis it may be postulated that the obstruc-

tion is caused by marginating leukocytes of the existing capillary channels, and by compression or even obliteration of some channels by the marked enlargement of the interstitial space at the expense of the capillary lumina.

Reparative Processes. Soon after injury to a tissue, the reparative processes are initiated. In response to the injury, cells tend to respond in a characteristic manner. Epithelial cells may proliferate and may thus produce the characteristic epithelial crescent. Endothelial cells in repair tend to proliferate and express this by capillary buds of granulation tissue. In the glomerulus, where the formation of new capillaries does not occur, the proliferat[illegible] of endothelium is inhibited and is not prominent. As in most inflammatory processes, the connective tissue reaction is very prominent. The interstitial connective tissue cells proliferate and lay down fibrils of connective tissue substance in the interstitial spaces. In the glomerulus the majority of the new connective tissue fibrils are laid down in the interstitial space, primarily in the area of the stalk or mesangium (Figs. 12, 13, and 14). The epithelial and endothelial basement membranes are frequently thickened by the deposition of additional basement membrane substance. In some examples of chronic glomerulonephritis the thickened basement membranes may show a reticulated or honey-combed appearance with the periodic acid-silver stain suggesting the irregular deposition of connective tissue substances between the epithelial and endothelial basement membranes. The capillary bed of the glomerulus may be permanently obliterated by contracture of pericapillary connective tissue and as a result of the death of endothelium of collapsed capillaries (Fig. 19).

Subacute Glomerulonephritis

Progressive subacute glomerulonephritis, as one might expect from the clinical story, shows changes in common with both acute and chronic glomerulonephritis. Capsular epithelial proliferation is often striking. The glomerular tufts usually show glomerular scarring and shrinkage, but often there are evidences of active inflammation in the form of neutrophils and edema. It is of interest that the periodic acid-silver stain which does not stain fibrin, does react with the delicate fibrils of the epithelial crescents in both acute and subacute glomerulonephritis. Thus it is apparent that these fibrils cannot be fibrin threads as some observers have suggested, but are fine connective tissue fibrils.

Lipoid Nephrosis

In the "pure" disease occurring in children, the glomerular lesion has caused confusion. Generally it is agreed that minor degrees of

glomerular scarring are present and thickened basement membranes have been described as the primary change.[10] In the present study it was believed that the most striking findings in juvenile lipoid nephrosis were the increase in the number of interstitial or mesangial cells and the irregular increase in connective tissue in the glomerular stalk (Fig. 15). It is believed that these changes reflect a minor reparative, proliferative process in response to a slight glomerular injury as the clinical picture of the disease would suggest.

Allen[9] placed lipoid nephrosis and eclampsia in a group of membranous glomerulonephritis. He expressed the paradox that these diseases, suggesting a relatively mild glomerular involvement clinically, show far more change in the glomerular basement membrane than is found in full-blown acute glomerulonephritis, which, he stated, usually shows little change. From the present discussion it can be seen that there is no paradox. In acute glomerulonephritis there is tremendous edema and inflammation in the connective tissue space between epithelium and capillary endothelium, while in lipoid nephrosis and eclampsia the change in this connective tissue is extremely mild. The correlation between structural change and clinical picture is excellent.

Chronic Glomerulonephritis

Glomerular scarring may become severe as a result of very intense acute glomerulonephritis, acute glomerulonephritis passing into progressive and active subacute or chronic glomerulonephritis, repeated episodes of subclinical acute glomerular inflammation, or perhaps by a slowly progressive chronic disease without any significant acute phase. Three general types of glomerular scars can be seen: the atrophic scar, the moderate scar, and the hypertrophic scar, although intermediate types will occur. Generally these are all present in one kidney, but one type is usually predominant.

The atrophic scar suggests glomerular collapse and capillary obliteration with little connective tissue production (Fig. 16). These scars are often surrounded by a thick hyaline crescent suggesting that in this instance they may represent the end stage in organization of a glomerulus involved with marked capsular epithelial proliferation. The atrophic scar is relatively non-specific and is seen in vascular nephritis and pyelonephritis.

The moderate scar shows definite deposition of connective tissue in the connective tissue spaces of the glomerulus in both a pericapillary and stalk location. Both the epithelial and endothelial basement membranes are thickened and are often separated by a thin layer of edema fluid so that a double contoured effect is produced in the pericapillary

zone. Contraction, distortion, and simplification of the glomerular lobules result from this scarring. Much of the capillary bed is obliterated. Adhesions between the tuft and Bowman's capsule are common. The glomerular epithelium frequently assumes a pseudotubular appearance when several of these adhesions divide the capsular space into numerous pockets (Figs. 17 and 18).

The hypertrophic scar is the result of tremendous connective tissue proliferation in the tuft (Fig. 19). The lobules of the tuft are enlarged and assume a clubbed shape. The connective tissue proliferation is most marked in the center of each lobule, but pericapillary fibrosis is often marked, resulting in very thick-walled capillaries. The scar in the center of the lobule often shows focal hyalinization of the fibrillar scar. When predominant, this glomerular lesion produces a picture which may superficially resemble diabetic glomerulosclerosis, but may be distinguished by the cellularity of the central scar, the pericapillary scarring, and the absence of the marked hyaline arteriosclerosis of the arterioles and the glomerular root. Allen[9] designated the lesion showing this change as predominantly chronic lobular glomerulonephritis, and pointed out the occurrence of a nephrotic syndrome with it.

Acute exudative and proliferative changes from a terminal acute exacerbation of glomerulonephritis may be engrafted on glomerular scars of these three general types. Fibrinoid change in afferent arterioles and rare focal areas in glomeruli may be due to an exacerbation of acute glomerulonephritis or, more likely, to the severe hypertension associated with some cases. It is believed that a malignant phase of hypertension may complicate chronic glomerulonephritis.[9]

The end result of all glomerular scarring, of whatever cause, is, of course, a non-specific hyaline ball.

Resolution

Several investigators[9,10] have indicated that 50 per cent or less of patients with acute glomerulonephritis die eventually of renal failure. What is the process in those who have a clinically complete recovery and never subsequently show signs of their acute attack? Logical explanation must depend on understanding the disease process. In the early phase of acute glomerulonephritis the changes seen are those of exudation, edema of the tuft, and polymorphonuclear and mononuclear infiltration. Following this early exudation is the initiation of the proliferative processes of repair. As in any inflammatory reaction, the severity of the injury and its duration determine the degree of connective tissue proliferation which in turn determines the degree of

eventual scarring. One may expect, therefore, the extremes—mild injury, exudation, resolution of exudate with practically no connective tissue response or severe and prolonged injury, prolonged exudation, marked reparative processes, and eventual marked distorting scarring. To make this concept workable one must assume that most of the mononucleated cells in the glomerulus in acute glomerulonephritis are histiocytic and may, therefore, migrate from the glomerulus with subsidence of inflammation. If they were connective tissue cells or endothelial cells, it is difficult to see how a return to near normal would be possible. It is apparent that resolution must occur in the glomerulus in a manner somewhat comparable to the process in lobar pneumonia.

It appears probable that the three general types of glomerular scars of chronic glomerulonephritis represent the result of variations of severity and duration of injury, exudation, and repair. One may postulate that the atrophic form represents an acute destructive process with little reparative reaction. The moderate scar may represent a prolonged course of mild injury and repair, while the hypertrophic scar suggests a prolonged, moderately severe injury with tremendous connective tissue proliferation.

What is the prime site of injury in acute diffuse glomerulonephritis? If it were the endothelium, the striking finding primarily should be thrombotic phenomena. Glomerular capillary thrombosis does occur commonly with sepsis, bacterial endocarditis, myocardial infarction, and other diseases.[11] Here the disease must be considered as predominantly one of endothelial injury. Acute focal thrombotic glomerulonephritis[10] is perhaps the best name for this nephritis due to endothelial injury.

If the epithelium were the prime site of injury in acute glomerulonephritis, we would expect to see primarily necrosis, degeneration, and desquamation of epithelial cells, marked adhesions between the tuft and Bowman's capsule, and crescent formation with little underlying inflammation of the glomerular tuft. In only rare cases does the formation of crescents and adhesions occur with slightly involved glomerular tufts, while the opposite is the rule.

The connective tissue may well be a primary site of injury in the glomerulus. Some support for this hypothesis is gained from the findings in experimental glomerulonephritis produced in hypersensitivity reactions. Arteritis and cardiac valvulitis characteristically accompany glomerulonephritis in many of the experimental procedures. Thus, in this type of hypersensitivity reaction we see relatively selective injury to vascular connective tissue adjacent to endothelium. Certain simi-

larities between glomerulonephritis and rheumatic fever give additional support to this hypothesis. In both glomerulonephritis and rheumatic fever there is a peculiar relationship to hemolytic streptococci. Each disease may show a characteristic latent period between the bacterial disease and the development of its acute manifestations. Rheumatic fever is almost purely a disease of connective tissue but yet it produces injury to the mesothelium secondary to inflammation in the underlying pericardial connective tissue. From the foregoing morphologic data on glomerulonephritis, experimental hypersensitivity phenomena, and the similarities to rheumatic fever, it may be inferred that the glomerular connective tissue (basement membranes, ground substance, fibrillar connective tissue, and fibroblastic cells) is the primary site of injury. Whether this injury is a direct physiochemical alteration of the connective tissue substances or whether it is directed toward the fibroblast is not known.

Summary

The normal glomerulus has an interstitial connective tissue space extending about the capillaries. This space is bounded by endothelial and epithelial basement membranes and is continuous with the space of the connective tissue stalk or mesangium which contains connective tissue cells.

In acute inflammation the processes of exudation, repair, and resolution occur in the glomerulus in a manner comparable to inflammation in other organs. Lipoid nephrosis is characterized by some deposition of connective tissue and proliferation of connective tissue cells in the glomerular stalk.

Chronic glomerulonephritis shows three general types of glomerular scars: the atrophic, the moderate, and the hypertrophic forms.

The primary site of injury in the glomerulus in diffuse glomerulonephritis is believed to be the connective tissue.

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[*Illustrations follow*]

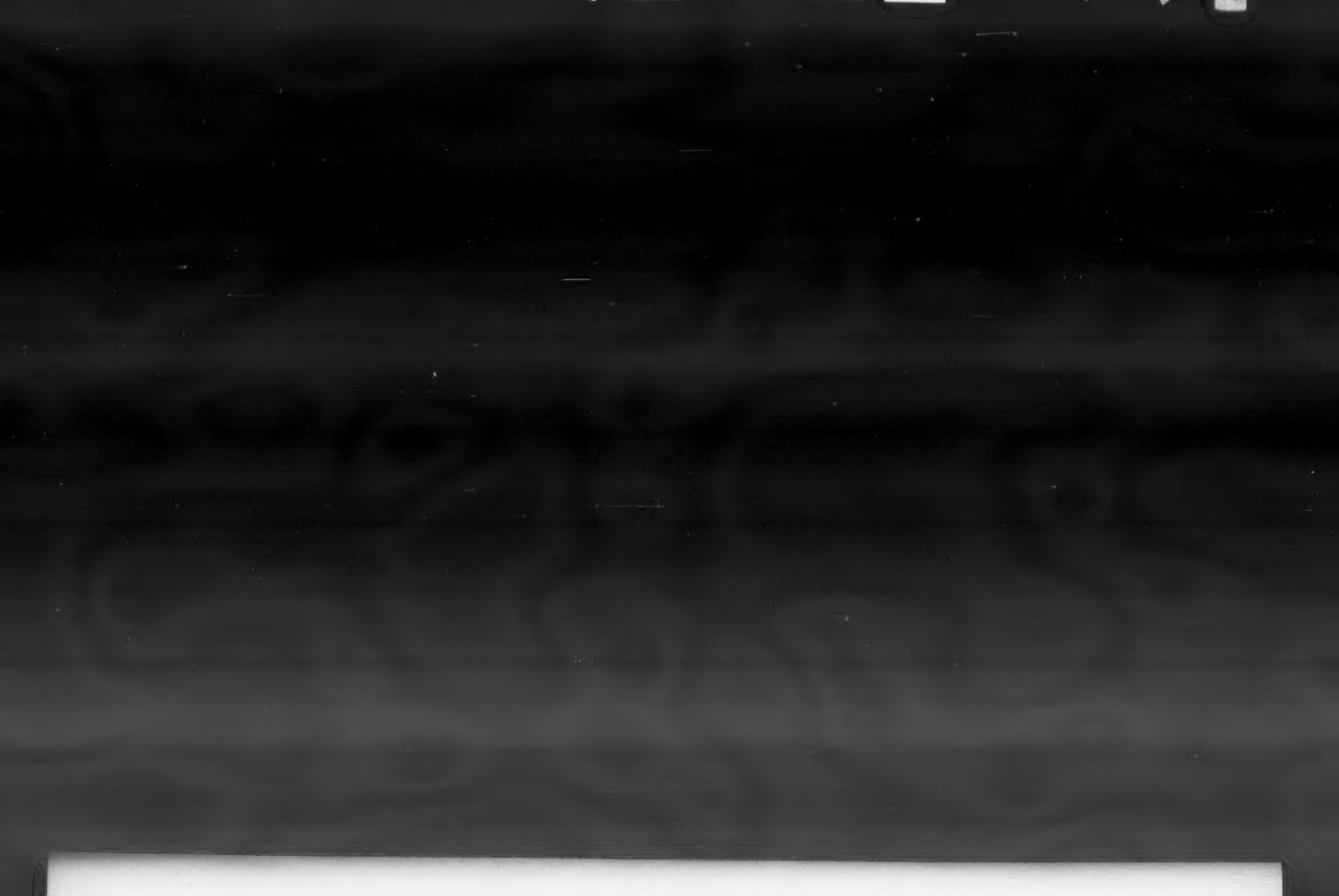
LEGENDS FOR FIGURES

FIG. 1. A lobule of a normal glomerulus with adjacent structures. Tangential sections produce an apparent focal thickening of the capillary walls. Periodic acid-silver methenamine stain. × 850.

FIG. 2. The lobule in Figure 1 with certain features labelled. Ep = epithelial nucleus; En = endothelial nucleus; F = the fibroblast-like connective tissue cell nucleus in the mesangium.

FIG. 3. A glomerular lobule in a case of sepsis due to *Staphylococcus aureus*. In this lobule ballooning edema has begun to separate capillaries and their basement membranes from the epithelial basement membrane. Periodic acid-silver methenamine stain. × 850.

FIG. 4. The lobule in Figure 3. In the center of the lobule one capillary is completely detached from the epithelial basement membrane. The other three capillaries still maintain some degree of attachment. Ep = epithelial nucleus; En = endothelial nucleus; C = capillary; R = red blood cell; P = polymorphonuclear neutrophilic nucleus; F = either fibroblast-like or histiocytic nucleus.



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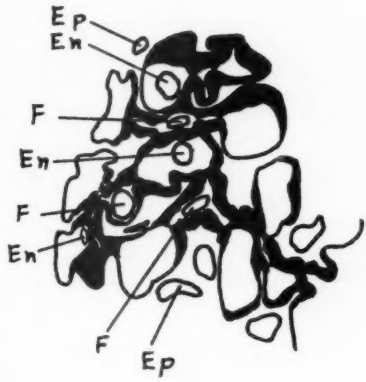

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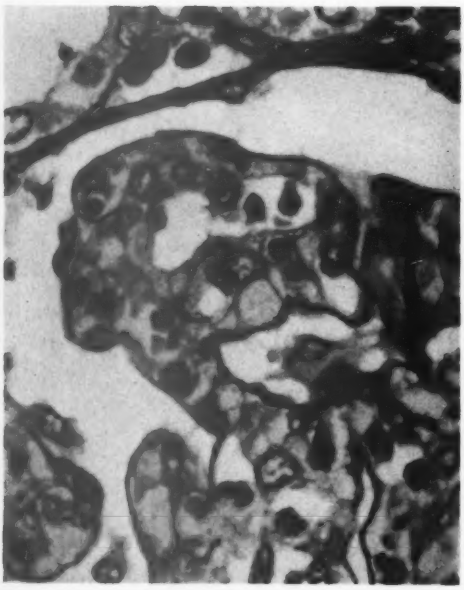

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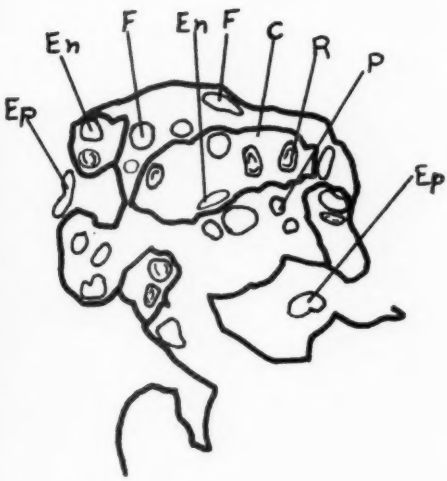

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FIG. 5. Acute exudative glomerulonephritis. The stages of leukocytic margination. Periodic acid-silver methenamine stain. × 850.

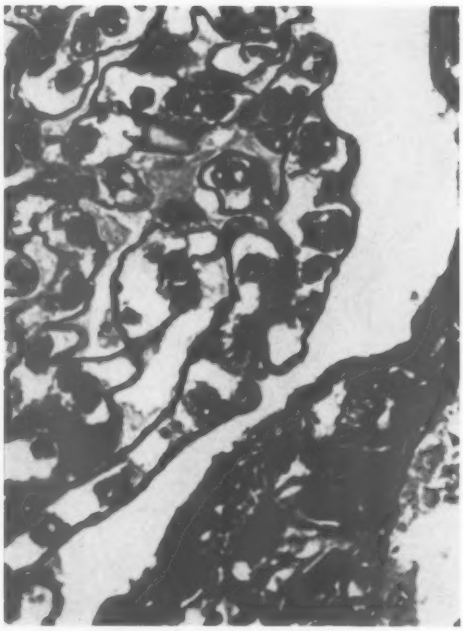
FIG. 6. The same as Figure 5. Ep = epithelial nucleus; En = endothelial nucleus; M = monocyte; F = fibroblast-like connective tissue cell nucleus; P= polymorphonuclear neutrophilic nucleus.

FIG. 7. Acute exudative glomerulonephritis. In this glomerular lobule ballooning edema of the interstitial space is prominent. Migration of neutrophilic leukocytes into this interstitial tissue has occurred. Periodic acid-silver methenamine stain. × 850.

FIG. 8. The same as Figure 7. En = endothelial nucleus; C = capillary; P = polymorphonuclear neutrophilic nucleus; F = either a fibroblast-like nucleus or a histiocytic nucleus; L = lymphocyte.

FIG. 9. Acute exudative and proliferative glomerulonephritis. Ballooning edema and leukocytic migration are present. Periodic acid-silver methenamine stain. × 850.

FIG. 10. The same as Figure 9. The Y-shaped nucleus labeled F is a histiocytic nucleus which appears to be distorted by ameboid motion. P = polymorphonuclear neutrophilic nucleus; En = endothelial nucleus; C = capillary; R = red blood cell; F = histiocytic or fibroblastic nucleus.



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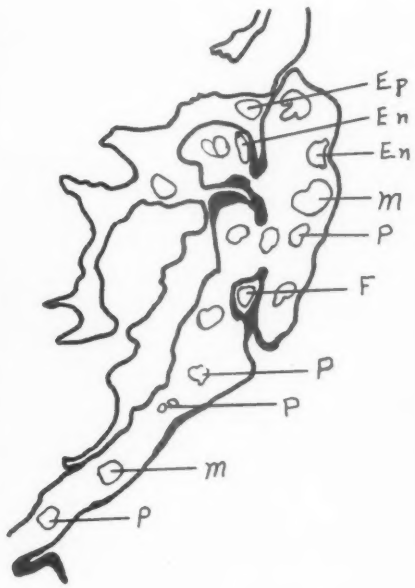

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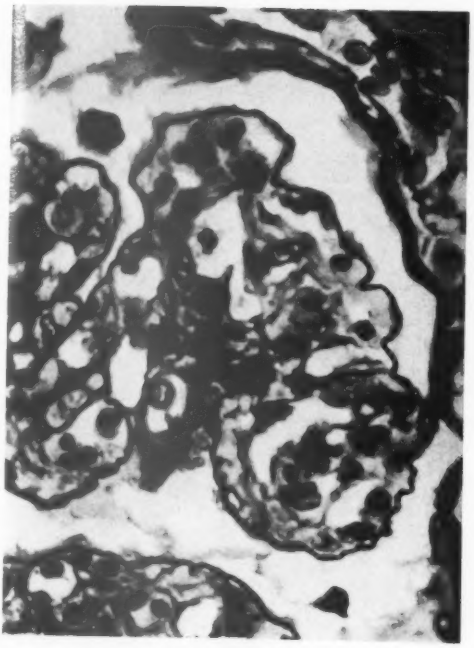

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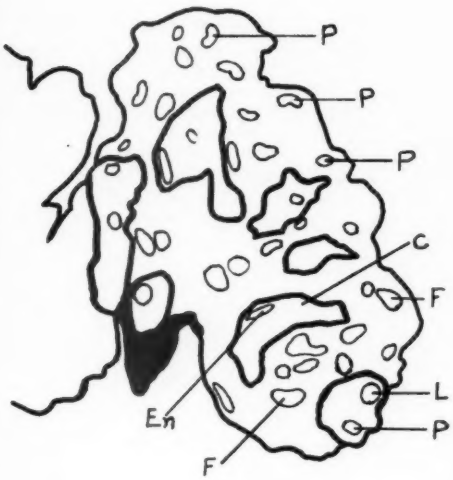

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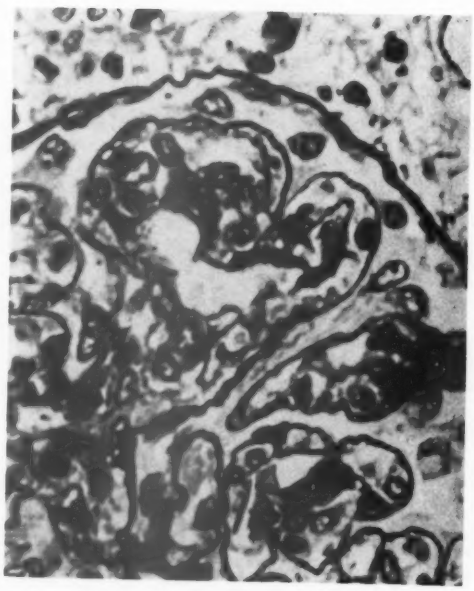

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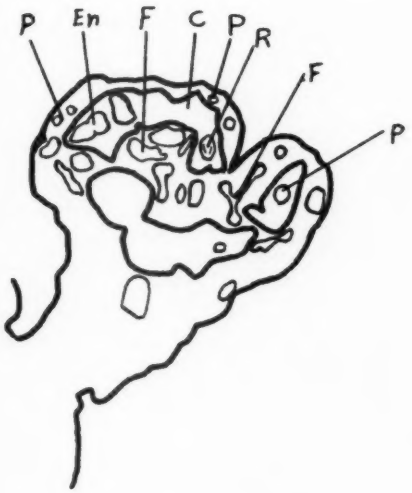

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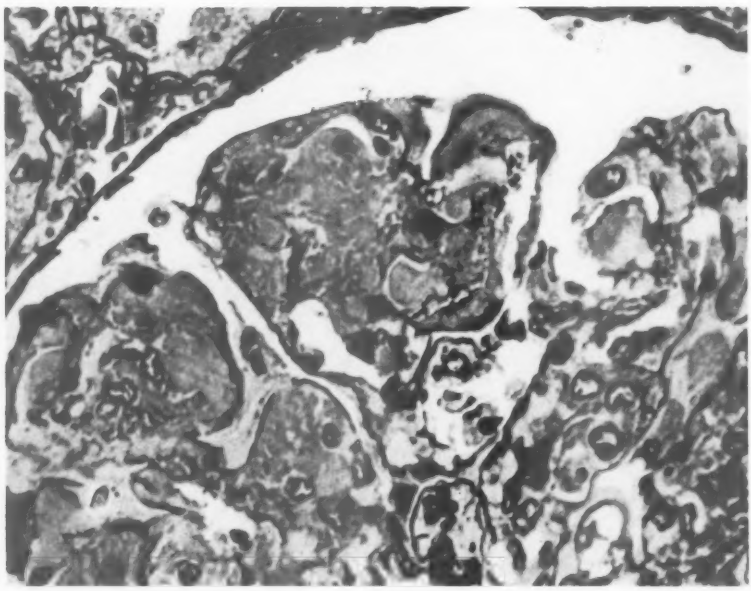
FIG. 11. Acute glomerulonephritis with focal deposition of fibrinoid material. The fine, black connective tissue fibrils within the fibrinoid substance do not show well in photomicrographs. Periodic acid-silver methenamine stain. × 850.

FIG. 12. Acute proliferative glomerulonephritis. Periodic acid-silver methenamine stain. × 850.

FIG. 13. Same as Figure 12. In this lobule the deposition of reparative connective tissue fibrils is beginning. For clarity only a portion of these fibrils are included. P = polymorphonuclear neutrophilic nucleus; En = endothelial nucleus; R = red blood cell; Ep = epithelial nucleus; F = either fibroblast-like or histiocytic nucleus.

FIG. 14. Acute glomerulonephritis of 12 weeks' duration. Prominent connective tissue scarring and cellular exudation may be seen together. The connective tissue deposition is primarily in the center of each lobule. Periodic acid-silver methenamine stain. × 191.

FIG. 15. Lipoid nephrosis in a child. The prominent glomerular stalks are filled with connective tissue cells. Periodic acid-silver methenamine stain. × 813.



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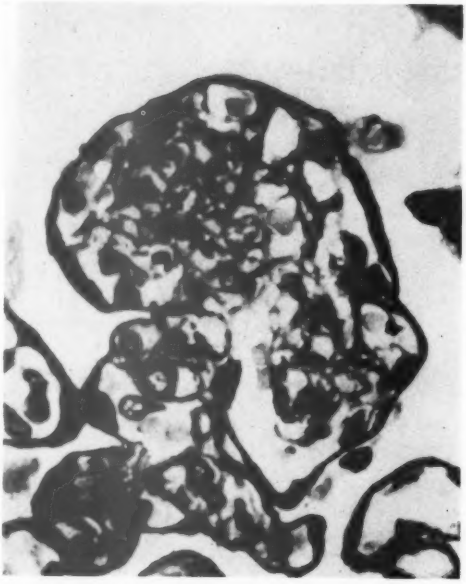

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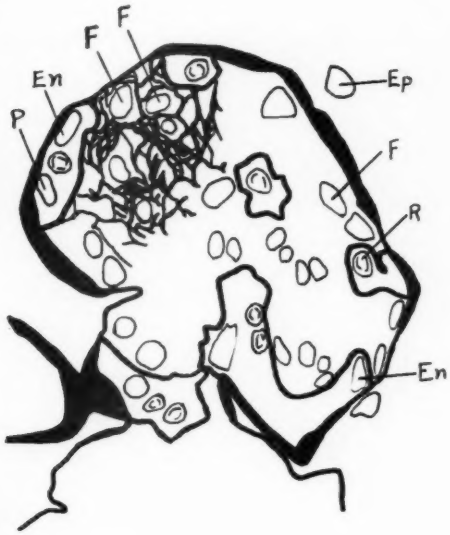

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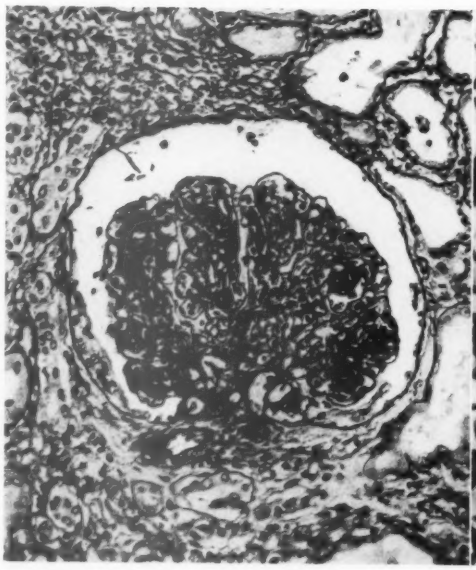

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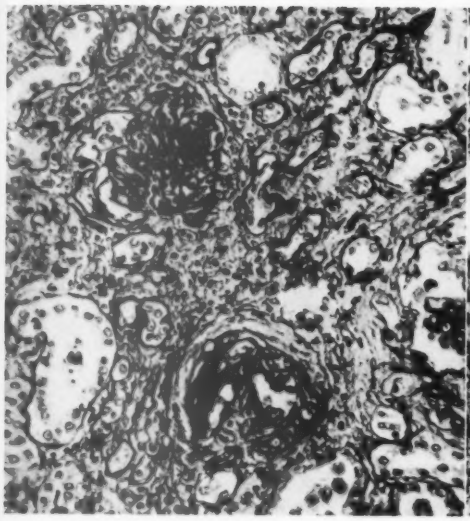
FIG. 16. Chronic glomerulonephritis. Atrophic glomerular scars. Periodic acid-silver methenamine stain. × 180.

FIG. 17. Chronic glomerulonephritis. Moderate scar. Periodic acid-silver methenamine stain. × 180.

FIG. 18. Chronic glomerulonephritis. Moderate scar. There is a pseudotubular structure about the scarred glomerular tuft. Periodic acid-silver methenamine stain. × 180.

FIG. 19. Chronic glomerulonephritis. Hypertrophic scar. There is extreme scarring, both in the centers of the lobules and about persistent capillaries. Periodic acid-silver methenamine stain. × 180.



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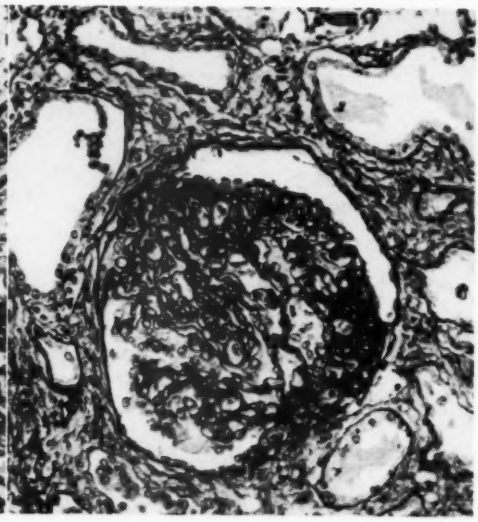

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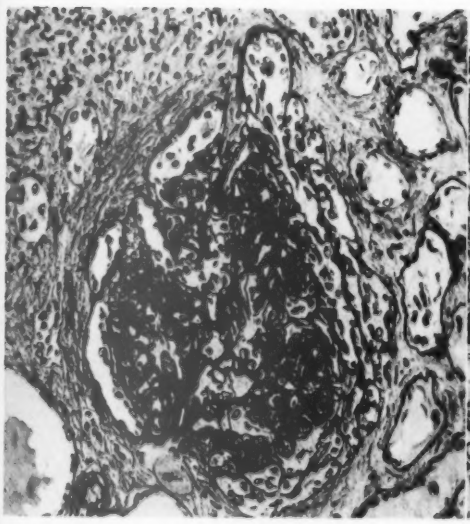

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SARCOID AND SARCOID-LIKE GRANULOMAS
A Study of Twenty-Seven Post-Mortem Examinations *

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The cases classified in the literature as sarcoid and sarcoidosis represent a heterogeneous group both clinically and pathologically. The difficulty of characterizing sarcoid may readily be seen by examining the definition of this disease process prepared by the Conference on Sarcoid of the National Research Council:[1]

"Sarcoidosis is a disease of unknown etiology. Pathologically it is characterized by the presence in any organ or tissue of epithelioid cell tubercles with inconspicuous or no necrosis and by the frequent presence of refractile or apparently calcified bodies in the giant cells of the tubercles. The characteristic lesions may be replaced by fibrosis, hyalinization or both.

"Clinically, the lesions may be widely disseminated. The tissues most frequently involved are lymph nodes, lungs, skin, eyes and bones, particularly of the extremities.

"The clinical course usually is chronic with minimal or no constitutional symptoms; however, there may be acute phases characterized by a general reaction with malaise and fever. There may be signs and symptoms referable to the tissues and organs involved.

"The intracutaneous tuberculin test is frequently negative; the plasma globulins are often increased.

"The outcome may be clinical recovery with radiographic evidence of residue, or impairment of function of organs involved, or a continued chronic course of the disease."

In the present study an effort is made to clarify the relationship among the sarcoid and sarcoid-like granulomas seen in 27 necropsied cases collected at the Armed Forces Institute of Pathology.

Classification and Analysis

The cases for this study were selected from the following groups: (1) with undiagnosed non-caseous granulomas, 13 cases; (2) with granulomas of sarcoidosis, 10 cases; (3) with granulomas of sarcoidosis with clinical and pathologic evidences of secondary tuberculosis, 4 cases. Twenty-three patients were male and 4 female. This discrepancy in sex distribution is related to the predominance of males in the Army, reflected in the material in the files of the Armed Forces Insti-

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tute of Pathology. The ages ranged from 18 to 68 years. Seven of the 27 patients were Negroes.

The granulomas usually could be readily classified as either tuberculoid or foreign body type. The tuberculoid granulomas (Fig. 1) were characterized by a large number of epithelioid cells and by relatively few giant cells. The foreign body granulomas (Fig. 2) were characterized by many giant cells and by the paucity or absence of epithelioid cells. It was often difficult, however, to assign a granuloma to one or another of these categories since all degrees of variation between the typical forms were noted. In some instances granulomas of both types were seen within the same organ and within different organs of the same patient.

The giant cells contained inclusion bodies of several types: (1) plate crystal slits—the so-called cholesterol slits (Fig. 3), (2) asteroid or spiculated bodies (Fig. 4), (3) Schaumann bodies—the round or oval non-crystalline laminated inclusions (Fig. 5), (4) non-lipid crystalline material (Fig. 6). On this basis it was possible to classify the granulomas into four groups according to the type of inclusion bodies found in the giant cells.

The cases are classified and summarized in Table I. Three of the cases (nos. 1, 25, and 26) have already been reported,[2,3] and several of the remaining cases were included by Ricker and Clark[1] in their study of 300 cases of sarcoidosis. Two of the cases (nos. 19 and 20) have been reported by Pinkerton and Iverson[4] as probable cases of histoplasmosis.

Group I consists of only 2 cases with granulomatous lesions of the foreign body type in the lungs. The giant cells contained asteroid bodies and large plate crystal slits. Although in case 1 the granulomas were extensive, in neither this case nor in case 2 could death be attributed directly to them. Because the lesions were present only in the lungs, it was impossible to exclude an exogenous etiologic agent.

In the 12 cases of group II (nos. 3 to 14) with giant cells containing only asteroid bodies, granulomatous lesions were observed in a variety of organs. Although most of these lesions were of foreign body type, in 5 cases granulomas of tuberculoid type were noted also, sometimes in almost equal number. A few giant cells containing small plate crystal slits were present in cases 10, 12, and 13 of this group, but they were not as prominent as in the cases of group I. In only one patient (case 9) could death be attributed to the granuloma, since no other cause could be found to account for the terminal hemoptysis. Neither sarcoidosis nor tuberculosis was suggested clinically in any

of the cases. A disturbance of fat metabolism was noted in 7 of the 12 cases.

Giant cells containing both asteroid bodies and Schaumann bodies were observed in the 7 cases composing group III (cases 15 to 21). The granulomatous lesions were of both the foreign body and tuberculoid types and they appeared in many organs. A few giant cells in 6 cases contained small plate crystal slits, but these were not as prominent as in the cases of group I. The probable cause of death in 3 cases was the granuloma or terminal tuberculosis. No evidence of any relationship to metabolic disturbances was noted in cases of this group. A clinical diagnosis of sarcoidosis was made in case 17. In case 18, although there was some caseation necrosis of the epididymis, liver, and kidneys, repeated tuberculin tests were negative. In cases 19 and 20 there was a serum hyperglobulinemia. Tuberculin tests were not done in either case 19 or case 20.

In one of the 7 cases there was morphologic or biologic evidence of tuberculosis in addition to the non-specific granuloma. In this patient the signs and symptoms of disease became apparent 1 year before death. Several tuberculin tests were negative, and smears of the sputum showed no acid-fast bacilli. Five months before death there was an exacerbation of the disease, all of the previous Mantoux sites suddenly became positive, and subsequent tuberculin tests were positive. At about the same time acid-fast bacilli were demonstrated in the sputum and in a left axillary mass, and, shortly before death, in the spinal fluid. At necropsy, caseation necrosis was observed in lungs, liver, and spleen, and in some bronchial and para-aortic lymph nodes. The lobes of the lung and the lymph nodes that contained the asteroid bodies and the Schaumann bodies and crystals did not show caseation necrosis. Acid-fast stains of several tissue sections of non-necrotic bronchial lymph nodes revealed no acid-fast bacilli.

Pinkerton and Iverson,[4] in a more recent study of cases 19 and 20, observed large numbers of fungi, probably *Histoplasma capsulatum*, in the necrotic adrenal glands. The organisms were difficult to identify with the usual tissue stains, but were readily seen when periodic acid-Schiff's reagent was used. They concluded that these 2 cases probably represent uncomplicated histoplasmosis.

Granulomatous lesions were observed in almost all organs in the cases of group IV (cases 22 to 27) characterized by giant cells containing only Schaumann bodies. These lesions were of both foreign body and tuberculoid type, although the tuberculoid type predominated. In cases 24, 26, and 27 a few giant cells contained small plate

TABLE I

Classification and Analysis of Twenty-seven Cases of Sarcoid and Sarcoid-like Granulomas

Case no.	A.F.I.P. no.	Race, sex	Age	Clinical diagnosis	Organs and tissues† containing granulomas	Tissue acid-fast stains	Necrosis	Type of‡ granuloma	Possible disturbances in fat metabolism	Special tests	Cause of death
					Group I. Asteroid bodies and large plate crystal slits within giant cells						
1	203190	W F	68	Tuberculosis or carcinoma	Lung *SL	–	–	F			Cocaine reaction
2	94944	W M	68	Coronary occlusion	Lung *SVR	–	–	F	Obesity		Coronary thrombosis
					Group II. Only asteroid bodies within giant cells						
3	184035	C M	22	Methyl alcohol poisoning	Lung * Liver *E Spleen *	– – –	– – –	F			Acute methyl alcohol poisoning
4	181599	W M	30	Pulmonary embolism	Lung *VR Lymph nodes (hilar)	–	– –	F	Obesity; fatty infiltration of liver		Pulmonary embolism
5	120711	W F	55	Carcinoma of cecum or regional ileitis	Lung *V Spleen (?)		– –	F	Obesity; hypothyroidism; fatty infiltration of liver		Acute suppurative ileitis
6	95602	W M	44	Coronary occlusion	Pancreas *	–	–	F	Obesity; pancreatic necrosis; fatty infiltration of liver		Acute hemorrhagic necrosis of pancreas
7	124086	M		Cerebral malaria (?)	Lung *	–	–	F	Fat embolism following trauma		Pulmonary and systemic fat embolism
8	109562	W M		Appendicitis	Lung *VR Liver *VR Spleen	– – –	– – –	F			Ruptured appendix
9	149058	W M	22	Hemoptysis, cause undetermined	Lung * Spleen *R Lymph node (bronchial)* Liver *	– – 	– – – –	F	Fatty infiltration of liver	Sputum, acid-fast stains, negative	Hemoptysis, possibly due to granuloma
10	106812	C M	22	Generalized hemorrhage	Spleen *SRE Lymph nodes *VRE Lung R Liver R	– –	– – – –	Ft			Purpura

11	74926	W	F	62	Intestinal obstruction with perforation	Spleen * Lung Lymph nodes * Liver	– –	– – – –	Ft		Tuberculin, negative; animal inoculation, negative	Intestinal obstruction
12	109226	W	M	34	Unexplained sudden death	Lung *S Heart Spleen Liver	–	– – – –	FT			Coronary thrombosis
13	96128	W	M	49	Myocardial infarction	Lung SR Lymph nodes *VR Liver VR Spleen E Adrenal rest tumor Heart(?) Thyroid gland(?)	– – –	– – – – – – –	FT	Hypothyroidism		Myocardial infarction
14	91001	W	M	47(?)	Diabetes mellitus	Lung *VR Spleen Liver (?) Thyroid gland(?)	–	– – – –	Ft	Diabetes mellitus		Diabetic acidosis
					Group III. Asteroid bodies and Schaumann bodies within giant cells							
15	113763	W	M	20	Accidental death	Lymph nodes *SVR Lung OR Liver	– –	– – –	Ft			Trauma
16	85448	W	M	28	Suicidal death	Lung *VR Lymph nodes *SVRE Kidney *VRE Spleen VRE Liver SVRE Heart OE	– –	– – – – – –	FT			Trauma
17	96016	C	M	26	Sarcoidosis	Lung OCVR Lymph nodes *SOVRE Spleen RE Liver	–	– – – –	Tf		Protein, normal; x-ray of extremities, negative; tuberculin, negative	Granuloma
18	169363	W	M	27	Malignant hypertension; undiagnosed generalized granuloma	Lymph nodes *V Lung *L Kidney OVE Lymphatics (ureteral and hepatic) Epididymis Liver Diaphragm Stomach Pons	–	– – + – + + – – –			Protein 6.2 with A/G 3.8/2.4; tuberculin, negative	Granuloma

TABLE I (Continued)

Case no.	A.F.I.P. no.	Race, sex	Age	Clinical diagnosis	Organs and tissues† containing granulomas	Tissue acid-fast stains	Necrosis	Type of‡ granuloma	Possible disturbances in fat metabolism	Special tests	Cause of death
Group III. Asteroid bodies and Schaumann bodies within giant cells (*Continued*)											
19	121747	W M	54	Chronic glomerulonephritis, pulmonary tuberculosis	Tongue SOVL	−	−			Protein, 9.5 with A/G 5.2/4.3; animal inoculation, negative; (*H. capsulatum* demonstrated in adrenal gland)	Histoplasmosis
					Lung *SOCVRL		+				
					Stomach SOL		−				
					Kidney O	−	+				
					Spleen E	−	+				
					Adrenal gland	−	+	Ft			
					Liver E		−				
					Lymph nodes		−				
					Heart		−				
					Thyroid gland		−				
					Pancreas		−				
					Pectoralis muscle		−				
20	119987	W M	30	Miliary tuberculosis	Lymph nodes SOCR	−	−			Protein, 9.7 with A/G 5.1/4.6; x-ray of extremities, negative; (*H. capsulatum* demonstrated in adrenal gland)	Histoplasmosis
					Spleen *SVR	−	−				
					Lung OCRE	−	−				
					Liver SOC		−	FT			
					Adrenal gland R	−	+				
					Rib R	−	−				
					Kidney O	−	−				
21	148970	C M	30	Tuberculous meningitis	Lymph nodes (bronchial) OCVR	−	−			X-ray of extremities, negative; tuberculin negative, later positive; spinal fluid, acid-fast stained smear, positive	Granuloma; terminal tuberculosis
					Lymph nodes (bronchial and aortic) VR		+	FT			
					Lung *OVR		+				
					Liver		+				
					Spleen VR		+				
Group IV. Only Schaumann bodies within giant cells											
22	124456	W F	32	Regional ileitis, pulmonary embolism	Ileum OVR	−	−				Pulmonary embolism
					Liver CE		−	Ft			
23	173430	W M	18	Chronic cholecystitis	Lymph node OR		−				Cardiac failure during cholecystectomy
					Liver E	−	−	Tf			

24	172709	C	M	25	Sarcoidosis	Heart E	−	−			Protein, 6.4 with A/G 1.9/1; x-ray of extremities, negative	Granuloma
						Lung SLE	−	−				
						Lymph nodes	−	−				
						Liver	−	−				
						Brain		−				
						Pituitary gland		−				
						Thyroid gland L		−				
						Prostate		−	Tf			
						Pancreas E		−				
						Epididymis		−				
						Kidney OE		−				
						Psoas muscle(?)		−				
						Retina		−				
						Spleen		−				
25	203191	W	M	46	Coronary occlusion	Lymph nodes OC	−	−		Obesity		Coronary thrombosis
						Heart	−	−				
						Spleen	−	−				
						Liver		−	T			
						Pancreas		−				
						Kidney		−				
						Lung	−	−				
26	163480	C	M	26	Tuberculosis, lung	Lymph node (cervical) SOCR		−			Protein, normal; x-rays of extremities, negative; sputum acid-fast stains, positive	Granuloma; tuberculosis
						Lymph node (interbronchial and pancreatic) OC	−	+				
						Liver C		+	FT			
						Lung CL		+				
						Stomach		−				
27	151036	C	M	35	Unexplained sudden death	Brain R	−	+				Granuloma; probable tuberculosis
						Dura SOR	−	+				
						Lymph nodes R	−	−				
						Lung		−				
						Spleen		−				
						Liver R		−				

† Symbols indicate morphologic changes:
* = Asteroid body
S = Plate crystal slits
O = Schaumann bodies
C = Non-lipid plate crystals
V = Vacuoles in giant cells
R = Extracellular round spaces
L = Lipophages
E = Eosinophils

‡ Key to symbols:
F = Almost all foreign body type
f = Few foreign body type
T = Almost all tuberculoid type
t = Few tuberculoid type
FT = Equal numbers of foreign body and tuberculoid types

crystal slits, but they were not prominent. The only case in this group in which any relation to metabolic disease was suggested was an example of obesity. In 3 of the 6 cases the probable cause of death was the granuloma or complicating tuberculosis. By biopsy, a diagnosis of sarcoid was made in case 24, but no tuberculin test was done. Cases 26 and 27 had superimposed tuberculosis in addition to the non-specific granulomas. In case 26 the sputum was positive for tubercle bacilli, and at necropsy there was necrosis of the lymph nodes, lung, and liver. Caseation necrosis of the brain and dura was present in case 27, but no tubercle bacilli could be demonstrated.

It may be seen that the granulomas form a spectrum, and thus it is impossible to define clearly the lesions of any one group. The spectrum ranges from granulomas of foreign body type containing asteroid bodies, through those of mixed type containing asteroid bodies, Schaumann bodies, and non-lipid crystals, to granulomas of primarily tuberculoid type containing Schaumann bodies and non-lipid crystals. In groups I and II the granulomas were usually unexpected findings at necropsy and were unrelated to the cause of death. They occurred in a limited number of organs and were associated in some instances with abnormalities of lipid metabolism. In group IV the granulomas occurred in all organs and were not associated with any recognizable disturbance in lipid metabolism. In many of these cases death was directly attributable to the granulomas or to complicating tuberculosis. The intermediate group had some characteristics of both extreme types.

Because the morphologic features of the groups of granulomas demonstrated here overlap, it would appear impossible to define specifically the lesions of sarcoid and sarcoidosis.

Discussion

The cases in groups I and II (in which the giant cells contained asteroid bodies) are probably related to local or systemic abnormalities in lipid metabolism. This relationship was first perceived by Hirsch,[5] who pointed out that the asteroid bodies occur frequently in close association with plate crystal slits ("cholesterol slits"). Upon chemical analysis of splenic tissue containing such lesions he found large amounts of stearin and palmitin. Furthermore, he produced rosette-shaped crystalline inclusion bodies within giant cells of tubercle-like lesions in the lungs of rabbits by means of cholesterol-palmitin mixtures given intravenously. The crystals gradually became changed in the tissues so that they were no longer soluble in the usual fat

solvents. He postulated that asteroid bodies form in the same manner.

Previously reported studies of material from case 1 of this series[2] indicated that the lipid plate crystals were not cholesterol or an ester of cholesterol although they gave a positive Liebermann-Burchard reaction. It was concluded that the crystals were an unidentified lipid, probably a steroid. It was shown that the same lipid which crystallized in plates was capable of crystallizing in the shape of asteroids, suggesting that the asteroid bodies in the giant cells might be related to the plate crystals with which they were closely associated. However, since it could not be demonstrated that the asteroid bodies in the tissue were crystalline in structure, it may be postulated that the lipid crystals act only as a template which is later removed, leaving behind a telltale cytoplasmic reaction. This does not conflict with the recent observation of Cunningham[6] that the asteroid body itself is possibly protein in nature.

Since asteroid bodies within giant cells have been described in association with a variety of disease processes,[1,2,6-9] they probably are not disease specific.

Because of the confusion that exists in the classification of granulomas of this type it is suggested that descriptive terms be used in naming the granuloma,[9,10] for example, generalized (or localized) foreign body (or tuberculoid) giant cell granuloma with asteroid inclusions.

The cases of group III will be considered after those of group IV. The cases in group IV in which the giant cells contained Schaumann bodies, and in some cases non-lipid crystalline material, probably represent a heterogeneous group. Some of these might be classified as sarcoid and sarcoidosis. The inclusion bodies from 2 of these cases have been studied and reported in detail in a previous paper.[3] The crystals were shown to contain ferric iron but no silicon, calcium, or beryllium. They were thought to be comprised of inorganic material. The possibility was suggested that the intracytoplasmic crystalline material forms Liesegang rings,[11] and thus makes up the Schaumann body.

Lesions similar to these have been described in berylliosis,[12] asbestosis,[13] talcum powder granuloma,[14] and silicosis.[8] None of these substances is thought to be the causative agent in the granulomas reported here. When more is known about the nature of the inclusion bodies in these granulomas, it is probable that additional etiologic agents will be discovered.

The cases in group III in which the giant cells contained asteroid

bodies, Schaumann bodies, and in some cases non-lipid crystals are probably of the same type as the cases in group IV except that there is a resulting or coexistent local or systemic abnormality in lipid metabolism. The organisms of histoplasmosis have been demonstrated in 2 of the cases.[4] Although some observers have reported seeing intermediary forms between asteroid bodies and Schaumann bodies, no intermediary forms were found in any of these cases.

The cases in groups III and IV are morphologically most compatible with the definition of sarcoidosis advanced by the National Research Council. Few of the patients, however, exhibited the clinical manifestations accepted as criteria. In both of these groups the underlying disease was sometimes complicated by tuberculosis, much as silicosis is frequently complicated by tuberculosis.

Summary

Sarcoid and sarcoid-like granulomas were found at necropsy in a variety of organs and tissues in the 27 cases presented here. Although there was considerable overlapping, the cases were divided into four groups as follows: group I (2 cases) with asteroid bodies and large plate crystal slits within giant cells; group II (12 cases) with only asteroid bodies within giant cells; group III (7 cases) with asteroid bodies and Schaumann bodies within giant cells; group IV (6 cases) with Schaumann bodies and non-lipid crystalline material within giant cells. The possible significance of the various types of inclusion bodies was discussed. Reasons were given for supposing that the lesions of groups I, II, and III were related wholly or in part to local or systemic disturbances in lipid metabolism, and that those of groups III and IV were more heterogeneous, some possibly related to those generally classified as sarcoid or sarcoidosis. Two cases in group III have more recently been diagnosed as histoplasmosis.

It was difficult to define clearly the lesions of sarcoid and sarcoidosis because the lesions formed a spectrum of granulomas.

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[*Illustrations follow*]

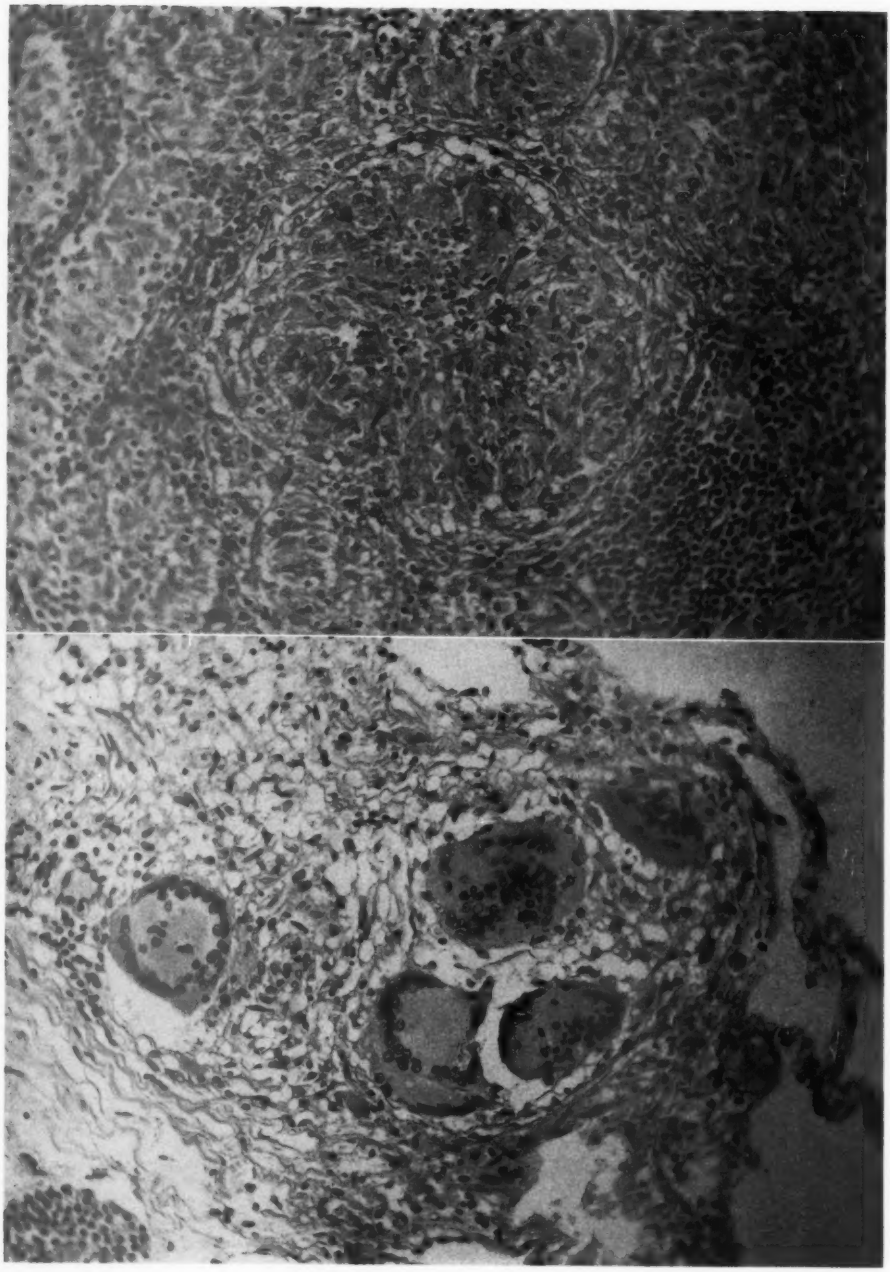
LEGENDS FOR FIGURES

FIG. 1. Granuloma of tuberculoid type. Spleen. Case 21. Hematoxylin and eosin stain. × 185. (Armed Forces Institute of Pathology neg. 103669.)

FIG. 2. Granuloma of foreign body type. Lung. Case 21. Hematoxylin and eosin stain. × 185. (A.F.I.P. neg. 103672.)

1

2

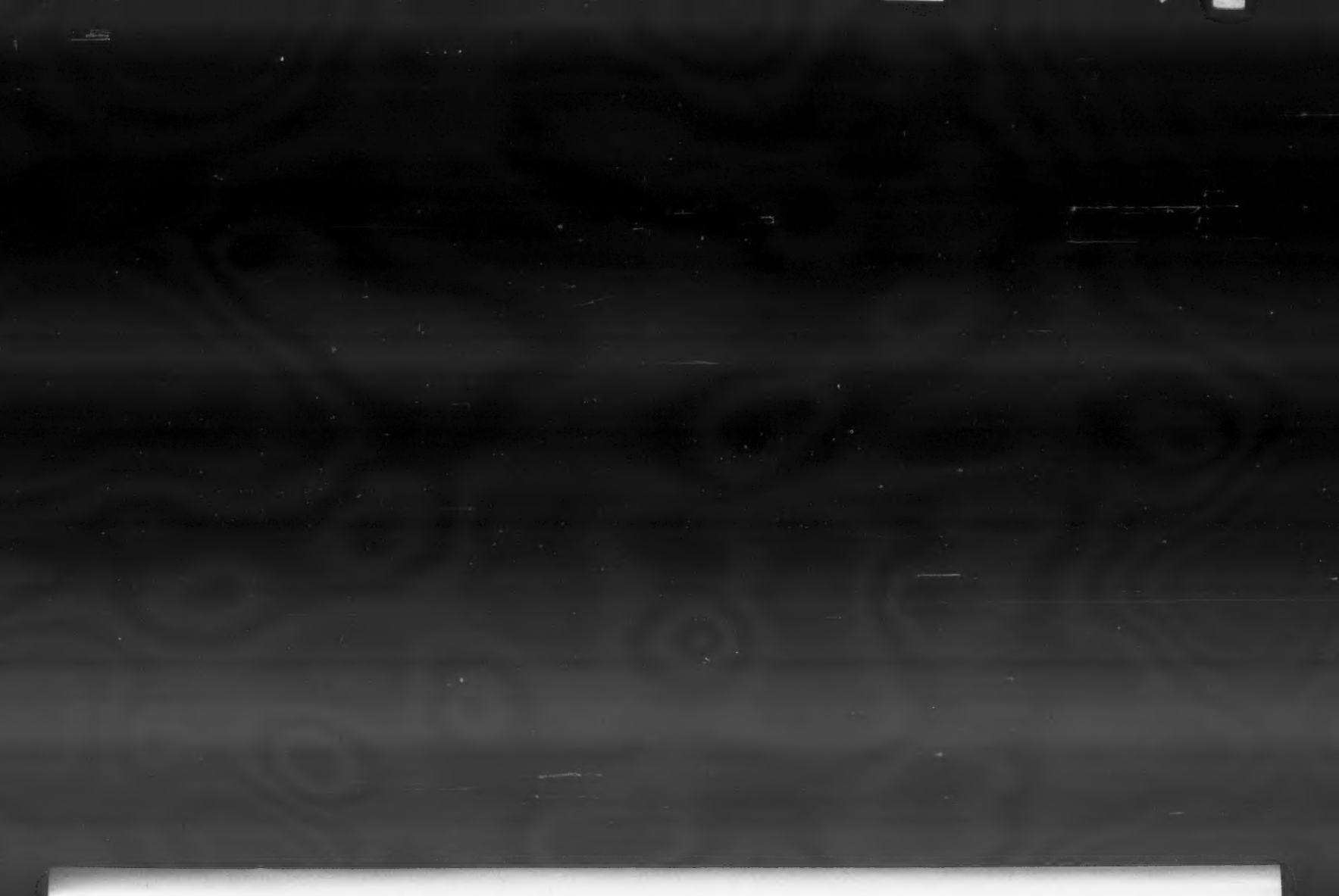


1

2

FIG. 3. Granuloma containing many plate crystal slits. Lung. Case 2. Hematoxylin and eosin stain. × 295. (A.F.I.P. neg. 86129.)

FIG. 4. Giant cell containing asteroid body. Lung. Case 16. Hematoxylin and eosin stain. × 900. (A.F.I.P. neg. 103515.)



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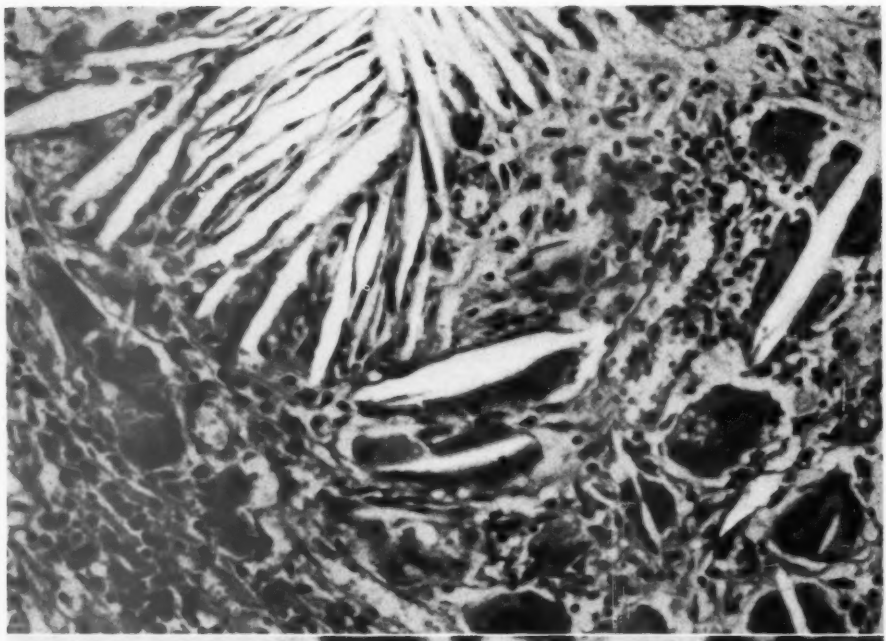

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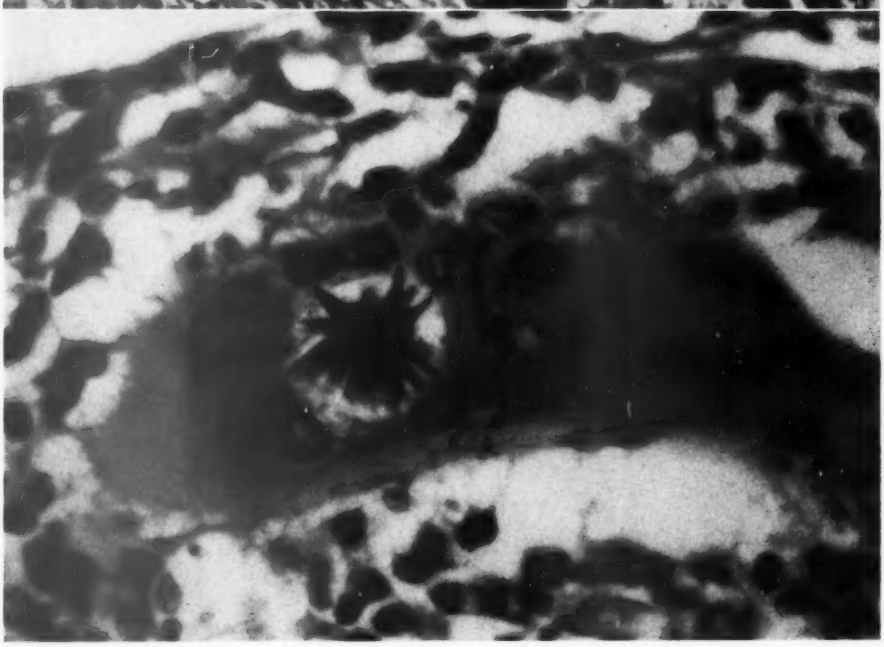

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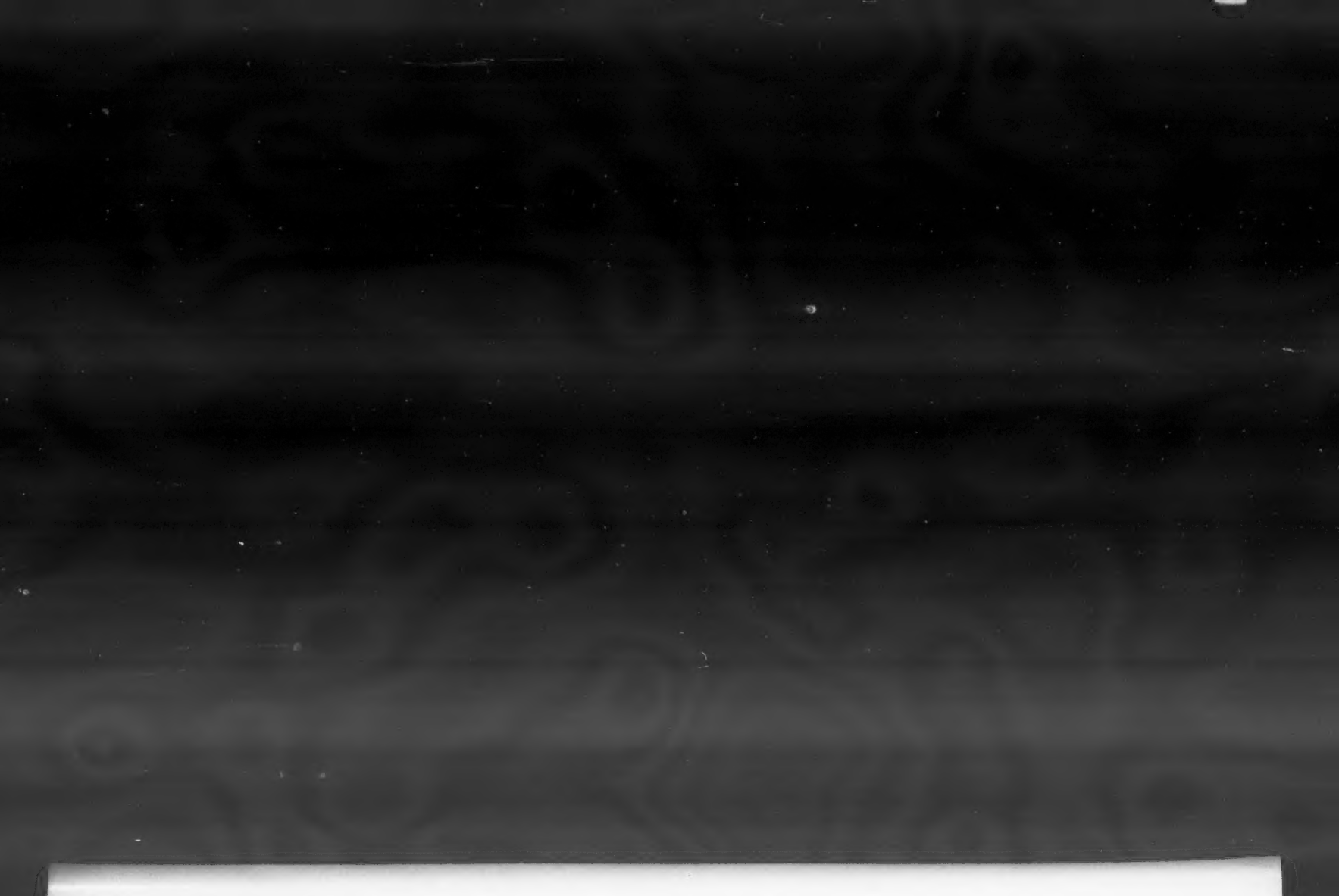
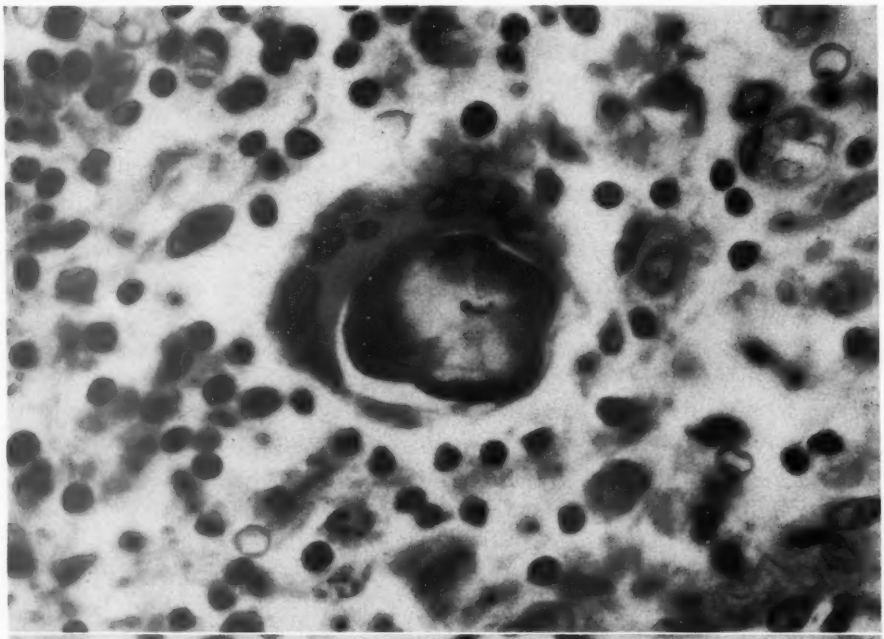
FIG. 5. Giant cell containing Schaumann body. Lymph node. Case 25. Hematoxylin and eosin stain. × 900. (A.F.I.P. neg. 103677.)

FIG. 6. Giant cell containing non-lipid crystals. Lymph node. Case 20. Hematoxylin and eosin stain. Polarized light. × 900. (A.F.I.P. neg. 104549.)





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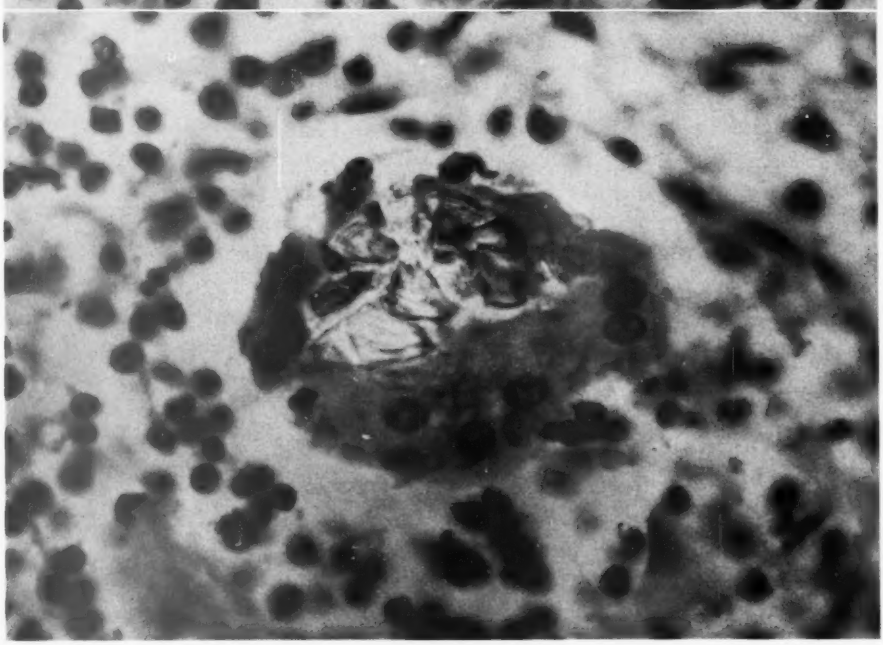

6

THE INFREQUENCY OF LIPID DEPOSITION IN SCLEROTIC VEINS *

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It is well recognized that the walls of veins may become thickened and fibrous with advancing age or when exposed to prolonged increase in intravascular tension. The changes are usually designated by the term phlebosclerosis. In a recent systematic study of the popliteal vein, Lev and Saphir[1] described this lesion. It is also apparent that the degree of sclerosis found in veins seldom approaches that of arteries in severity or extent. Whether or not phlebosclerosis and arteriosclerosis constitute essentially analogous processes is a matter of controversy. If the view is accepted that arteriosclerosis is a regressive lesion with many variable anatomical alterations of which none is of prime importance, there is little reason to regard phlebosclerosis as an independent process. By many observers, however, the deposition of lipid is considered to be an essential, early feature of the common type of arterial lesion generally termed atherosclerosis. Much evidence has been advanced to support each of these two conflicting interpretations.

If atherosclerosis results from lipid deposition, it would be necessary to demonstrate such deposits in veins in order to prove that phlebosclerosis is related to the common form of arterial disease. There appear to be some differences of opinion as to the frequency with which lipid deposits are found in veins.[2] The present study was undertaken to investigate this feature of phlebosclerosis. Varicose veins of the lower extremity were selected for examination, not only because of their availability due to the frequency with which they are excised, but also because these vessels are subjected to severe mechanical stress and show profound anatomical alterations. Moreover, in such incompetent varicose veins the blood pressure frequently approaches normal arterial pressure when the body is in an upright position.[3] Finally, phlebosclerosis related to advancing age has been described more frequently in the vessels of the legs than elsewhere in the venous system. If atherosclerotic changes occur in veins, they should most logically be found in varicosities of the lower limbs.

MATERIALS AND METHODS

Routine random histologic sections of 24 specimens of varicose saphenous veins and 4 post-phlebitic popliteal veins were available

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from the surgical pathology files of Bellevue Hospital. These were stained with hematoxylin and eosin, trichrome, and Weigert-van Gieson techniques. This material was fixed in Bouin's solution and preserved in 80 per cent alcohol and was therefore not suitable for fat stains. In addition, portions of 19 varicose saphenous and one postphlebitic popliteal veins received from the operating room were fixed in 10 per cent formalin. These measured from 20 to 45 cm. in length. Some had been turned inside out with the intima exposed during stripping. The rest were opened so that the intimal surface could be inspected. The vessels were stained grossly by immersion in Sudan IV for 3 minutes after dipping in 70 per cent alcohol. They were then rinsed in several washings of 70 per cent alcohol and returned to 10 per cent formalin. By this technique the adventitial fat is stained bright scarlet. Similar techniques have been used to demonstrate intimal lipid in arteries. The intima of the veins was examined under magnifying lenses for evidence of intimal lipid deposition. At least three frozen sections were cut from representative or suspected areas of each vessel and restained by the usual technique with Sudan IV.

Various age groups were included. Of the 48 specimens, at least 35 were from patients over 40 years old and 21 were from patients over 50 years of age. Varicosities were known to have been present longer than 6 years in 24 cases and longer than 11 years in 19. In 11 cases there were associated ulcers of the legs. The sex was recorded for 43 cases and of these 30 were men.

Results

Gross Findings

The lumina of the excised varicose veins were relatively small upon gross examination, indicating that a great deal of contraction had occurred during removal. This accounted for some of the increased thickness of the walls of the veins but there was undoubtedly a marked overgrowth of tissue. The walls of the veins measured up to 3 mm. in width and were many times thicker than in normal veins of comparable size. Many were even thicker than small muscular arteries. There was some unevenness in this overgrowth in different areas. It was somewhat more pronounced in the regions where tributary branches entered. Small, relatively thin-walled, aneurysmal outpouchings up to 1.0 cm. in depth were common. The walls of the veins were firm and inelastic. The intimal surfaces were smooth and pearly gray. In two instances, minute deposits of calcium were detectable grossly but the walls of vessels were never rigid or brittle. No intimal plaques and no

lipid deposits were found in any of the stained gross specimens. The valves of the veins were semilunar, bicuspid, and symmetrically formed. Their leaflets were extremely delicate. No evidence of valvular alteration resulting from incompetence could be made out.

In three veins, areas of thrombosis with organization were noted. In one of these, a fibrous cord-like structure, 7 cm. in length and 1.5 mm. in thickness, partly filled the lumen of a vein and was attached at several points to the adjacent wall. This was obviously an organized thrombus. After staining with Sudan IV, small focal collections of lipid up to 4 mm. across were found on the surface of this cord-like structure but not in its depths and not in the adjacent intimal surface of the vein itself.

Microscopic Findings

No trace of lipid was found in the intima or media of any of the Sudan-stained frozen sections prepared from various areas of the veins removed from 20 patients, although the adventitial fat stained as usual. In the paraffin sections of all 48 cases studied, there was no evidence of lipid deposition. No fat-laden phagocytes, cholesterol crystal spaces, or atheromatous deposits of any sort were found. There was no discrete intimal plaque formation, although in transverse sections the intimal thickening was often irregular. Some of the irregularity appeared to be due to contraction of the vessel wall.

The increased thickness of the wall of the vein was due to a marked hypertrophy of smooth muscle and a proliferation of connective tissue in all three layers. The smooth muscle hypertrophy was often pronounced and of a degree never found in any type of arterial lesion. The boundaries of the various layers were more readily identified in longitudinal than in cross sections.

The intima was usually thickened over wide areas and consisted of cellular fibrous tissue that frequently contained a heavy complement of smooth muscle. The cellular constituents tended to be longitudinally disposed and at right angles to the circular muscle groups in the media. In Weigert stains, delicate elastic fibrils were seen to extend through the intimal layer, but splitting or reduplication of the internal elastic lamella into several layers of equal thickness, a finding that is very common in arteries, was usually not observed. Complete hyalinization of the thickened intima was never noted.

The smooth muscle of the media was often greatly increased. The smooth muscle cells were sometimes arranged in small bundles separated by interlacing strands of connective tissue so that the media frequently had an almost lobulated appearance. This arrangement is

present in the normal media of veins but is usually indistinct because the amount of interwoven fibrous tissue is ordinarily scanty. In the hypertrophied media of varicose veins this pattern may be exaggerated by the overgrowth. In some instances of marked medial fibrosis, the smooth muscle cells were widely spread apart. The intramural nutrient vessels that normally penetrate the media of veins were also rendered more conspicuous in the walls of varicose veins. They could easily be traced in the fibrous tissue, coursing from the adventitia through the media to the subintimal zone. In two instances small deposits of calcium were found histologically. These simulated the very early stages of medial calcification or Mönckeberg's sclerosis of arteries.

The adventitia of varicose veins was also increased in thickness and compactness. While consisting largely of fibrous tissue, incomplete layers of smooth muscle often were found. These layers were readily distinguished from the medial muscle as they tended to course perpendicular to the medial muscle and more or less parallel to the intima.

In the single case of organized parietal thrombosis described grossly, compact groups of fat-laden phagocytes and extracellular lipid masses were found just beneath the surface of the thrombus, reproducing in every respect the histologic details of early intimal lipid streaks commonly found in arteries. The wall of the adjacent vein, however, contained no lipid. In one of the other two vessels with organizing thrombi, masses of extracellular lipid were noted. In this instance, however, the thrombus was incompletely organized and the lipid material was limited to the area of old thrombus that had not been converted into fibrous tissue.

Discussion

The histopathologic differences between sclerosis of varicose veins and of arteries are sufficiently pronounced to suggest that the mode of development of the two processes may be unrelated. The most striking difference is the regular absence of lipid deposition in veins. Lipid has been described in veins in other situations but it occurs either as very scanty deposits or under very exceptional circumstances. Lev and Saphir[4] noted small deposits of lipid in sclerotic iliac and popliteal veins but regarded this as a secondary phenomenon probably related to previous degenerative changes. Geiringer[5] found that the localized intimal fibrous plaque that is frequently found in the inferior vena cava near its point of origin from the iliac veins was sometimes faintly impregnated with lipid. He noted small fat droplets in the center of some lesions of this type. It should be pointed out that fat stains on hyalinized connective tissue, whatever its location, frequently reveal it

to contain a light film of stainable lipid. Thickened auricular endocardial plaques in rheumatic heart disease are often impregnated with lipid in this manner.

It is perhaps this finding of lipid as an obviously secondary manifestation in a variety of old scar tissues that provides one basis for the opinion that such deposits in arteries, although more pronounced, are nevertheless of the same nature. Arterial lipid deposits, however, occur early in the course of atherosclerosis before hyalinized fibrous tissue has formed and are found as large, free masses of lipid material such as are not ordinarily seen in association with hyalinized connective tissue in extravascular locations.

Apparently, true venous atherosclerosis can occur in exceptional cases. Benda[6] described this lesion in the pulmonary veins of a patient with hypercholesterolemia, chronic nephritis, thyroid tumor, and renal xanthomata. He also stated that small quantities of lipid are sometimes demonstrable in varices and in the portal vein in portal hypertension. He emphasized, however, that nothing equivalent to arterial atherosclerosis is ever found in veins. We have observed a single case of rather pronounced intimal lipid deposition in the main pulmonary veins (Figs. 11 and 12) of a young woman with severe mitral stenosis and severe diabetes. Because of this observation, the pulmonary veins in 10 other cases of severe mitral stenosis were inspected closely. In one of these, two minute flecks of intimal lipid measuring 2 and 4 mm. across, respectively, were found at points of branching in major veins at the hilum of the lung. This patient was a 45-year-old woman with no clinical evidence of diabetes and in whom no blood cholesterol studies had been done. In addition to mitral stenosis, there was massive thrombosis of the left auricle and the thrombi occluded the points of entrance of the main pulmonary veins. In the other 9 cases no gross or microscopic evidence of lipid deposition was found in the pulmonary veins. We have not been able to demonstrate lipid in the portal vein in cases with severe cirrhosis. In one cavernous angioma of a vertebra studied recently, there was marked intimal fibrosis of large venous channels but no obvious lipid deposition. It may be concluded that lipid deposition does not ordinarily play a significant rôle in phlebosclerosis.

The question may be raised as to whether the immunity of veins to lipid deposition under ordinary conditions depends upon the inability of lipid to enter the vein wall or upon the ease with which lipid can be transported through it and eliminated without deposition. Small lipid deposits are not unusual in the main pulmonary arteries of persons over 40 years of age. The resistance of veins to lipid deposition cannot,

therefore, be attributed to peculiarities of serum lipid composition in venous blood since these arteries transport venous blood. The relatively low blood pressure in veins probably contributes to the failure of lipid deposition. As already indicated, however, the blood pressure in varicosities of the lower limbs often greatly exceeds normal pulmonary arterial pressure in upright positions. The differences between arterial and venous pressures cannot, therefore, explain completely the refractiveness of veins to lipidosis. This is further indicated by the observation that organized thrombi within varicose veins may become impregnated with lipid while the vein wall itself is uninvolved.

It seems likely that differences in structure between arteries and veins that are maintained even after the vessels become sclerotic may explain in large measure the differences in susceptibility to lipid deposition. Filtration experiments on excised vessels[7] have shown that serum colloids, including lipoproteins, are able to pass through vein walls with considerably greater ease than through those of arteries. During the course of such artificial filtrations, lipid is usually deposited in the tissues of the arterial wall. In veins such deposits are either very scanty or absent.

It is of interest that the pulmonary vein is apparently the only one susceptible to atheromatous changes even if the unusual combination of abnormally high pressure and blood lipid levels is required to produce such lesions. The pulmonary veins do not differ strikingly in structure from other large veins although the medial muscle is often more compact and the elastica more fully developed. The unique feature of pulmonary veins that distinguishes them from all others is that they transport arterial blood. The paucity of intramural vascularity in the intima and inner medial layers of normal arteries has given rise to the belief that these vessels derive part of their nutrition by diffusion through the intimal surface. The question may be raised, therefore, as to whether vessels that transport arterial blood, including the pulmonary veins, are not so constructed as to receive fluid transudate directly from the lumen in contradistinction to vessels that carry venous blood. The rich intramural vascularity of systemic veins suggests that their nutrition may be maintained largely through the capillary circulation. This interpretation would support the theory that the development of atherosclerosis depends upon the penetration of lipid-containing fluid from the blood through the intimal surfaces.

Summary and Conclusions

A study of 4 popliteal veins removed following thrombophlebitis and of 44 varicose saphenous veins revealed marked thickening of all

three layers of vessel walls due to fibrosis and hypertrophy of smooth muscle. No evidence of lipid deposition was observed. Atheromatous lesions were found in an organizing thrombus within one of these veins but not in the wall of the vein itself. Analysis of the factors concerned in the development of phlebosclerosis suggests that this process is not analogous to arterial atherosclerosis. It is further suggested that the resistance of veins to lipid deposition may depend in large measure upon their structural characteristics rather than upon the hemodynamics of venous blood flow or upon alterations in the pattern of serum lipids of venous blood.

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[*Illustrations follow*]

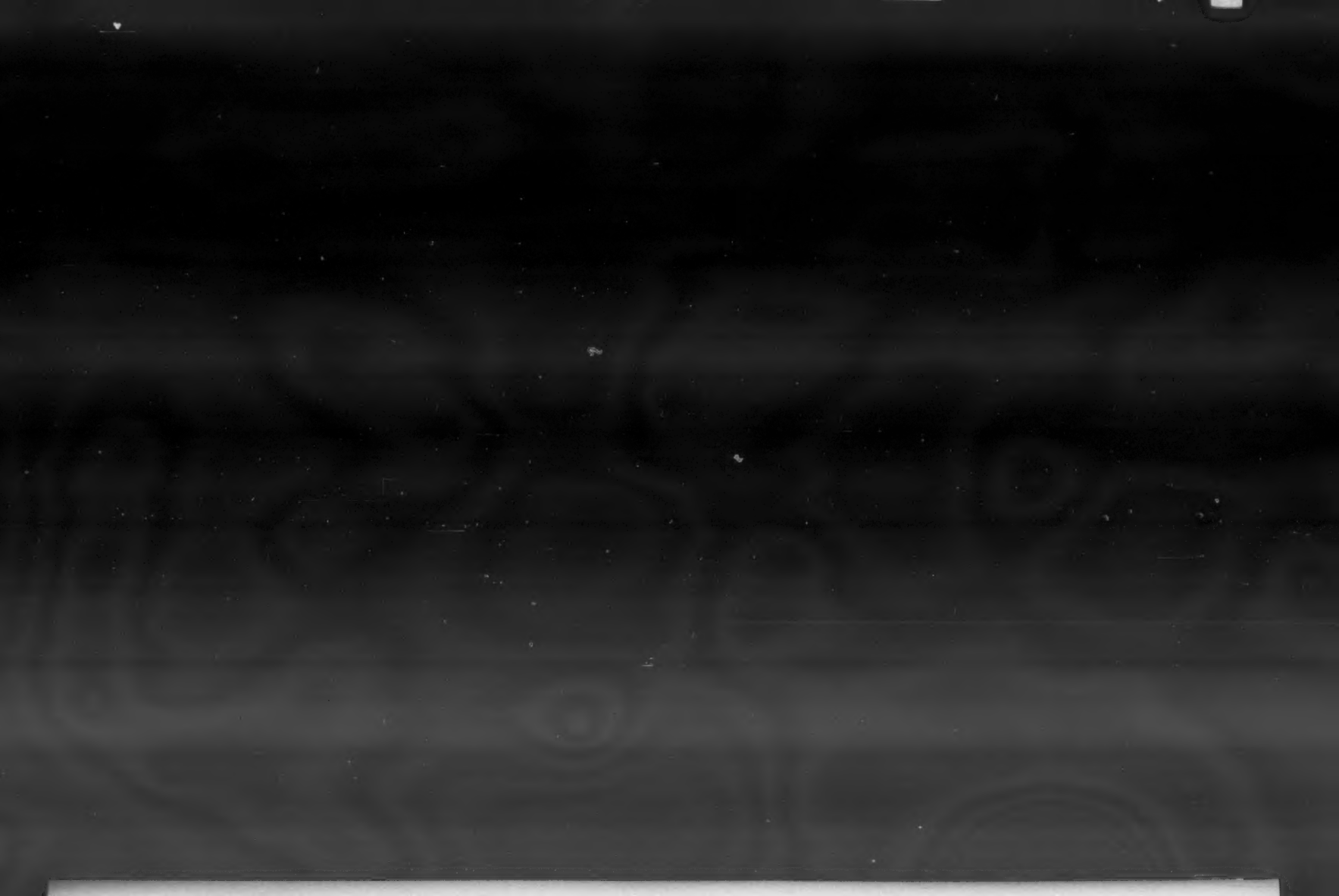
LEGENDS FOR FIGURES

FIG. 1. Marked thickening of the wall of a varicose saphenous vein. The light gray fibrous intima is uniformly thickened. Hematoxylin and eosin stain. × 15.

FIG. 2. Transverse section through the wall of a varicose vein. The thickened intima contains groups of longitudinal smooth muscle cells. The smooth muscle of the media is spread apart by fibrous tissue. The adventitia is very thick and fibrous. It contains groups of longitudinal smooth muscle bundles. Hematoxylin and eosin stain. × 90.

FIG. 3. The smooth muscle of the media is markedly hypertrophied. It is arranged in trabeculae separated by thin strands of connective tissue. Trichrome stain. × 86.

FIG. 4. The intimal smooth muscle is hypertrophied. It may be seen to course at right angles to the medial smooth muscle. Hematoxylin and eosin stain. × 90.



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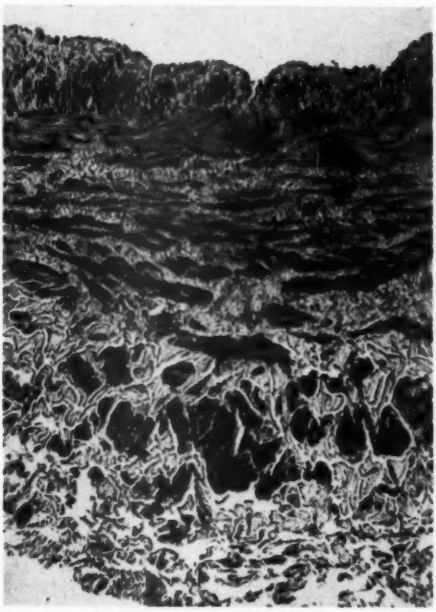

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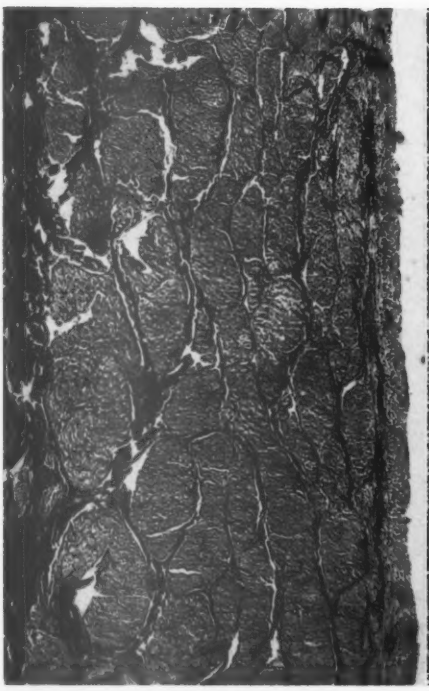

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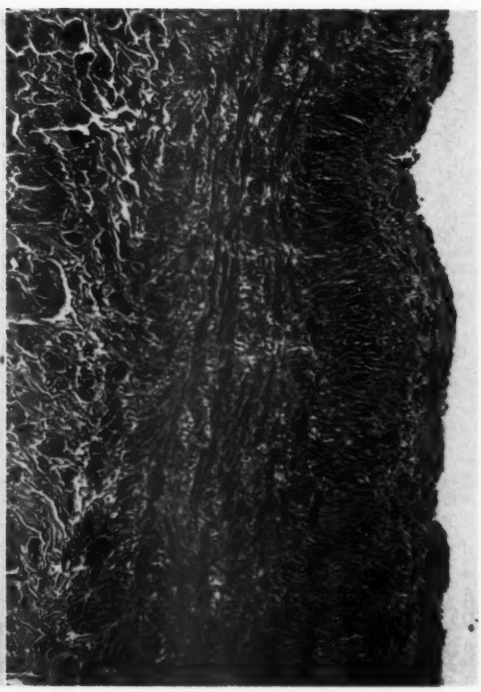

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FIG. 5. The smooth muscle cells of the media in this varicose vein are widely separated by fibrous tissue. The zones of transition of the three layers are obscured. Hematoxylin and eosin stain. × 134.

FIG. 6. Longitudinal section of varicose vein showing marked intimal fibrosis. Several penetrating blood vessels may be noted. Hematoxylin and eosin stain. × 115.

FIG. 7. Weigert's elastic tissue and van Gieson's stain. The internal elastica does not appear as a distinct lamella but is granular and frayed. Granular fragments of elastic tissue are present in the media. × 124.

FIG. 8. Weigert's elastic tissue and van Gieson's stain. The amount of elastic tissue at the junction of the thickened intima and media is increased but there is no splitting or reduplication of elastic fibers. Delicate elastic fibrils extend throughout the intima. × 153.

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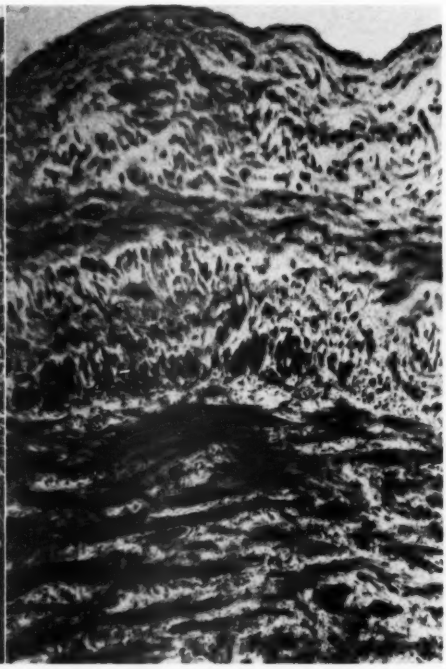

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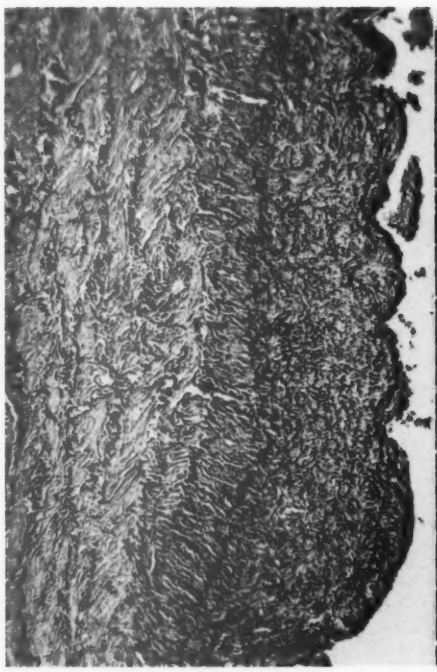

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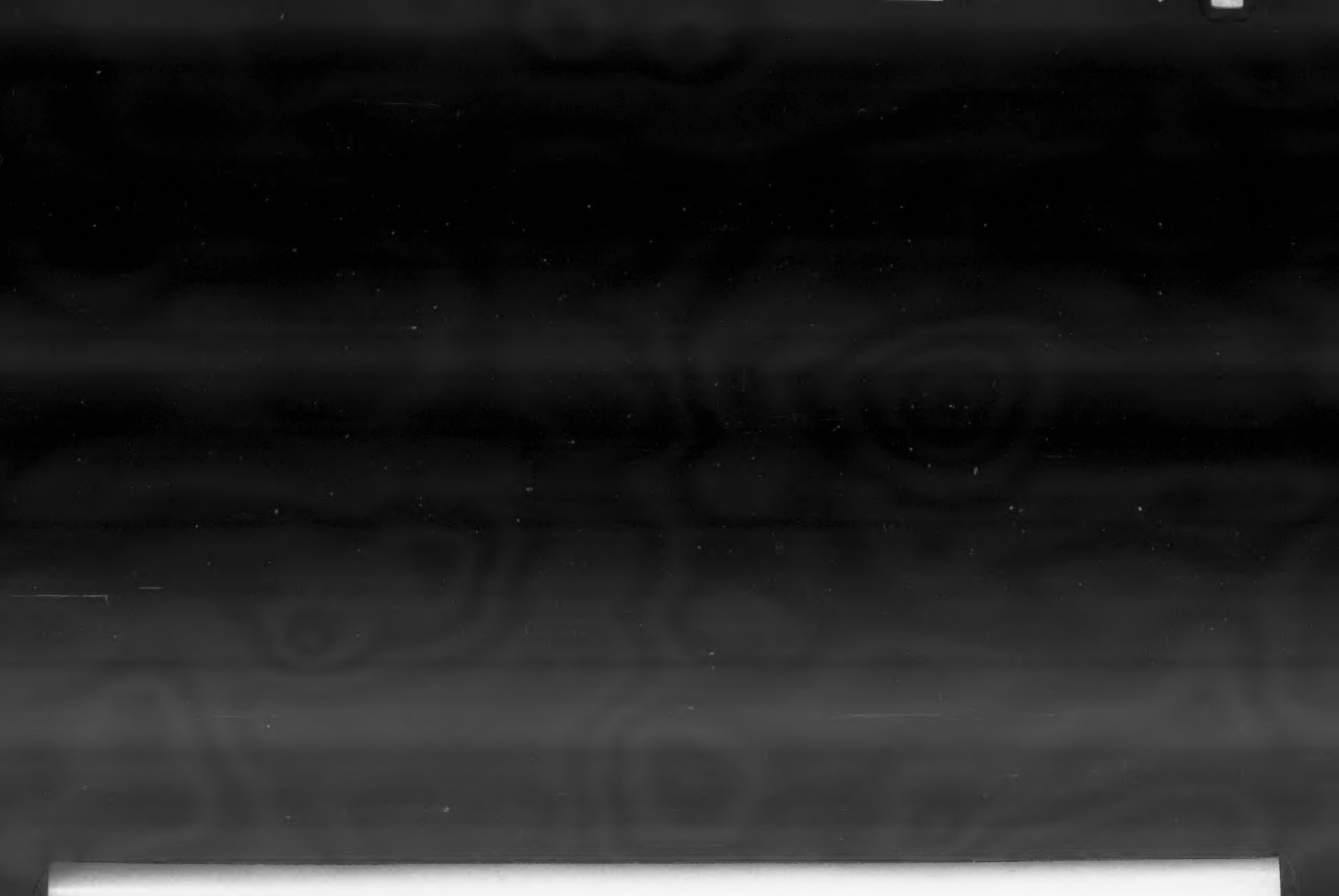
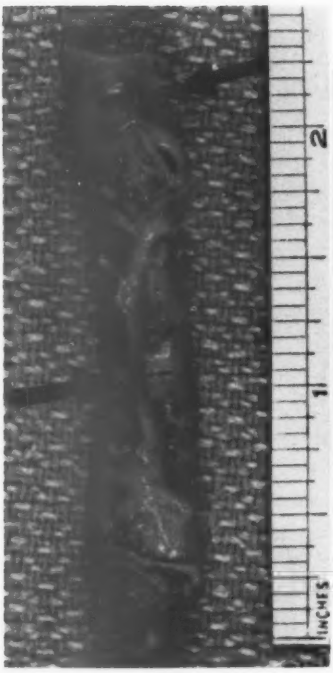
FIG. 9. Intimal surface of varicose vein stained with Sudan IV. A cord-like organized thrombus is attached to the intima at a number of points. The arrows indicate dark areas of lipid deposition in the thrombus.

FIG. 10. Cross section through the vein shown in Figure 9. The organized parietal thrombus is seen to fill the lumen partly. Near its surface is a dark area of lipid deposition. Frozen section, Sudan IV stain. × 7.

FIG. 11. Frozen section of a pulmonary vein from a 28-year-old woman with marked mitral stenosis and diabetes. The black material in the thickened intima represents lipid deposits. Sudan IV stain. × 115.

FIG. 12. Higher magnification of the vessel shown in Figure 11. Heavy deposits of lipid are present in the thickened intima. Rounded globules of fat also may be noted. Frozen section, Sudan IV stain. × 220.





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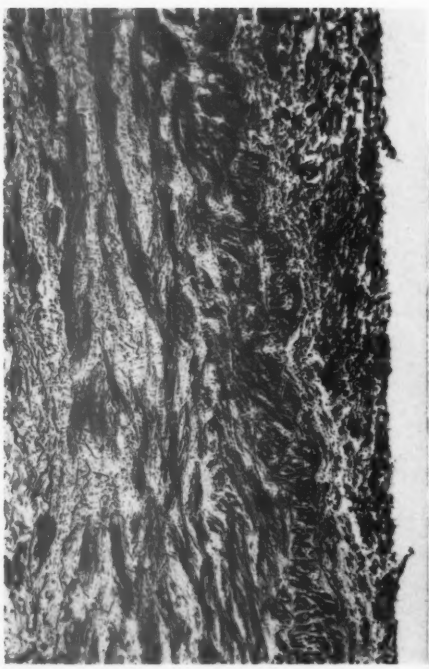

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RETICULAR PERITHELIOMA OF THE THYMUS *

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The classification of thymic tumors is a difficult problem for the pathologist. The controversy concerning the development of the thymus, the rarity of thymic tumors, and their unusual cytologic nature contribute to this confusion. Ewing[1] stated that "No group of tumors has more successfully resisted attempts at interpretation and classification than those of the thymus." There are a number of excellent case reports and reviews of thymic tumors[2-7] and valuable classifications are suggested.[8,9] The difficulty of distinguishing undifferentiated epithelial tumors from mesenchymal neoplasms adds to the complexity. At present there is a tendency to regard most thymic tumors as derived from endodermal reticular cells, and one classification is based wholly on this assumption.[9]

Many thymic tumors are described as showing "endothelial" or "perivascular arrangements" of spindle cells as well as the more definite epithelial cells. Such cases are presented by Poer,[10] Wu,[11] Mandlebaum and Celler,[12] Murray and McDonald,[13] and others. The terms "perithelial" and "hemangio-endothelial" are often used to describe tumors which are otherwise obviously of epithelial origin. This admixture of cellular components would seem to make classification as either epithelial or mesenchymal impossible. Nevertheless, the recognition that some types of thymic tumor are mesenchymal in origin may help to clarify this difficulty.

Recently seven mediastinal tumors have been studied in this laboratory. The cases presented a uniform structure which permitted classification as mesenchymal tumors.

REPORT OF CASES

Case 1

F. H., a 57-year-old male, was admitted with the complaint of "high blood pressure." A diagnosis of angina pectoris was entertained because of vague chest pains that occurred off and on for 12 years. Roentgenograms of the chest revealed an anterior mediastinal mass projecting slightly to the right of the midline, thought to be a dermoid. Thoracotomy exposed a large, irregular tumor in the upper, anterior mediastinum superior to the vena cava. The mass was completely removed by blunt dissection from the vessels except for a small pedicle which ran superiorly behind the manubrium in the midline. The postoperative course was uneventful. At no time in the patient's course was the diagnosis of myasthenia gravis considered.

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Gross Description. The surgically removed specimen consisted of an encapsulated, ovoid mass measuring 13 by 9 by 6 cm. and weighing 320 gm. A small amount of fibro-fatty tissue was adherent to the capsule. The capsule was translucent and pinkish gray with injected vessels. The mass was lobulated with areas of greenish blue discoloration. The cut surface of the tumor presented an indistinctly lobulated, variegated pattern. It was pale yellowish brown throughout, with numerous yellow-gray septa coursing through it. Small areas of hemorrhage were apparent as well as tiny yellowish flecks suggesting calcium. The tumor was of rubbery consistency. There were no cystic spaces, and the vascular nature of the tumor was not evident grossly.

Case 2

J. K., a 48-year-old, single, white female, was admitted with the chief complaint of urticaria. Hay fever and urticarial attacks had been frequent since birth. The patient had been hypertensive for many years without definite symptomatology. Roentgenograms of the chest showed a homogeneous density to the right of the midline overlying the base of the heart, thought to be a dermoid tumor. Exploration revealed a solid tumor, 5 cm. in diameter, loosely attached to the pericardium near the base and slightly to the right of the midline. A pedicle ran upward toward the thyroid gland and was ligated. The tumor was readily removed and had no attachments to the other mediastinal structures. The postoperative course was uneventful. At no time was the diagnosis of myasthenia gravis considered.

Gross Description. The surgical specimen consisted of a flattened, spherical mass weighing 75 gm. and measuring 3 by 5 by 5.5 cm. The external surfaces were pinkish gray with a small amount of fibro-fatty tissue attached to the smooth capsule. The cut surfaces revealed pinkish gray lobulated tissue in which numerous clefts, fissures, and septa were scattered. The lobules varied in size and bulged slightly. There was no gross evidence of invasion of the capsule by the tumor. There were occasional small foci of calcium, and these appeared to be chiefly at the periphery of the lobules. There were no areas of hemorrhage or cyst formation, and the vascular nature of the tumor was not evident grossly.

Case 3

L. L., a 39-year-old female who had had five previous admissions for treatment of chronic anemia, was admitted for the study of a mediastinal tumor. Three years prior to the date of operation she was told she had a chest tumor after routine roentgenograms, but she had refused operative treatment. The clinical impression was that the patient had acquired hemolytic anemia secondary to lymphoma, possibly Hodgkin's disease. Biopsy of lymph nodes and several sternal marrow studies failed to reveal a cause for the anemia. Surgical exploration of the mediastinal mass was advised. This procedure revealed a movable, encapsulated tumor held down superiorly by attachment to the sternum by loose adhesions. The tumor lay directly anterior to the aorta and pulmonary artery, and extended to the right and posterior to the lower portion of the innominate vein. There was no relation to other medias-

tinal structures. Postoperative recovery was uneventful. Subsequent studies revealed that the anemia had returned, and it was believed that the mediastinal tumor was incidental. At no time during the study of this patient was the diagnosis of myasthenia gravis entertained.

Gross Description. (Fig. 1). The surgically removed specimen consisted of a flattened, oval mass measuring 11 by 8.5 by 3.5 cm. and weighing 170 gm. The tumor was enclosed in a thin, fibrous capsule which was pinkish gray to red and covered by numerous tabs of loose areolar tissue. There was irregular lobulation of the surface of the tumor, and it was unevenly firm to palpation. The cut surfaces revealed deep yellowish brown lobules which bulged slightly and varied in size from 0.2 to 2.0 cm. in diameter. They were separated by fibrous bands of varying thickness which radiated outward from the capsule. There was no gross evidence of necrosis, cyst formation, or calcification, but there was a small area of hemorrhage beneath the capsule at one pole.

Case 4

B. D., a 57-year-old male, was admitted to the hospital in deep coma. The relatives stated that he was known to have had hypertension for many years. Recently he had experienced episodes of severe headache lasting several hours. Twelve hours before admission he had suddenly lost consciousness during an attack. Examination revealed that he had had a cerebral vascular hemorrhage. The patient died 48 hours after admission.

Necropsy Examination. Aside from massive hemorrhage in the right cerebral hemisphere, the gross and microscopic findings were of little interest except for a mediastinal tumor. There was an ovoid mass occupying the superior mediastinum and loosely attached to the basal pericardium and great vessels by areolar tissue. The mass was freely movable over these structures and moderately firm. The tumor was surrounded by a thin pinkish gray capsule which was vaguely lobulated. It measured 8 by 6 by 4 cm. The cut surfaces revealed many lobules of yellowish to pinkish gray tissue of varying size, separated by coarse and fine strands of fibrous tissue. The nodules varied from 0.8 to 3.5 cm. in diameter. There was no gross evidence of necrosis, cyst formation, or calcification. The vascular nature of the tumor was not evident grossly.

*Case 5**

A 47-year-old colored man was admitted with the complaint of abdominal pain. Strongyloidiasis had been diagnosed several years before, although the seriousness of the infection had not been appreciated. Roentgenograms of the chest revealed a mass in the anterior mediastinum over the base of the heart. The patient had bouts of severe diarrhea and abdominal pain, refusing all food and water. His condition became worse and he died on the fourth hospital day. The clinical picture had been explained on the basis of a malignant thoracic lesion with metastases.

* From the Mallory Institute of Pathology.

Necropsy Examination. A well encapsulated, pear-shaped tumor weighing 320 gm. was found in the anterior mediastinum to the left of the midline. The apex of this tumor was at the level of the sternomanubrial joint, and the base rested on the diaphragm. None of the adjacent structures were infiltrated or compressed. On section the surface was pink, meaty, and divided by thick fibrous septa into nodules from 3 to 4 cm. in diameter. Although on microscopic examination of the various organs numerous parasites were found, none was discovered in the mediastinal tumor. (See Kyle, McKay, and Sparling[14] for complete case report.)

*Case 6**

F. F., a 55-year-old, white, chimney sweep, was admitted with the complaint of a cough of 4 weeks' duration. He stated that he had been having episodes of chills and fever with intervening periods of normality for 3 days. He expectorated 1 cup of yellow sputum daily. Roentgenograms of the chest revealed an irregular density extending upward from the right hilus from the level of the third rib to the apex of the lung. At operation an encapsulated, lobulated, firm, yellow-gray mass 5 cm. in diameter was found anterior to the right lung root and hilum at the level of the superior pulmonary vein and resting on the pericardium. Pulmonary tissue taken for biopsy revealed chronic pneumonitis.

Gross Examination. The tumor measured 5 by 6 cm. and was completely encapsulated. The external surfaces were lobulated and pale pink. The cut surfaces were uniformly pale yellow-brown. The tumor was divided into lobules of varying size by bands of fibrous tissue.

Case 7

E. T., was a 43-year-old, white housewife who was admitted with complaints of dry cough, weakness, and a mass in the left chest. At her local hospital she had been found to be anemic (hemoglobin, 6.3 gm. per cent), and was given transfusion therapy 2 months before admission. Two weeks before admission she developed chills and aching chest pain. Roentgenograms revealed a well defined tumor mass in the anterior part of the left side of the chest. She was transferred to the Massachusetts Memorial Hospitals for surgical treatment. An exploratory operation revealed an encapsulated tumor of grapefruit size loosely attached to the left anterior-superior mediastinum. This was removed without unfavorable sequelae.

Gross Description. The surgical specimen consisted of an encapsulated tumor measuring 10 by 9.5 by 6.5 cm. and weighing 270 gm. The external surface was pale pink to dark red and lobulated. The cut surfaces were gray to yellow-brown and firm. The tumor was divided into lobules of varying size by interlacing, slender, gray-white, fibrous strands.

MICROSCOPIC STUDY

Multiple blocks from these tumors were fixed in Zenker's or formalin fixatives. Sections from each block were stained with phloxine

* From the Mallory Institute of Pathology.

and methylene blue or hematoxylin and eosin, phosphotungstic acid-hematoxylin, and Wilder's connective tissue reticulum stain. The tumors of cases 4 and 7 were stained with hematoxylin and eosin alone.

The microscopic appearances of these seven tumors were so similar that they may be described as a group. Minor variations from tumor to tumor will be mentioned.

The essential low-power architectural feature consisted of broad areas of tightly packed fusiform cells forming closely set knots and whorls grouped into lobules of varying size, which were separated by strands and broad bands of collagenous connective tissue. Under high power the cells, compactly grouped around a central capillary, formed somewhat indistinct, whorled bodies (Figs. 2 to 5). These densely cellular whorls fused intimately with each other, and, being cut in different planes, produced a variegated pattern. There was no suggestion of broad, undulating columns or strands as seen in some perineural fibromas, ordinary fibromas, or leiomyomas.

In addition to this cellular pattern, there were variable numbers of lymphocytes. A few lymphocytes were always present, and in at least one block from each of the tumors they occurred in sheet-like masses. These areas may be interpreted as remains of the thymic cortex since Hassall's corpuscles were absent and moderate numbers of large pale cells with vesicular nuclei were present. These latter cells were not associated with silver staining reticulum fibers and may be thymic epithelial cells. The tumor cells seemed to be enveloping and replacing the thymic cortex in some areas. Where these lymphocytes were present in considerable numbers the fusiform tumor cells were inconspicuous. Even there, however, they could be recognized as widely separated by the lymphocytes and arranged loosely about the blood vessels (Figs. 6 and 7).

The individual tumor cells were best studied in areas where edema had separated them slightly. They were elongated and fusiform, terminating in two or three delicate processes. The faintly eosinophilic cytoplasm was undifferentiated and contained no visible granules or fibrils. Phosphotungstic-acid hematoxylin stain revealed only homogeneous, bluish brown cytoplasm. The nuclei also were elongated or cigar-shaped with blunt ends. The nuclear membranes enclosed a small amount of fine, powdery chromatin. The dust-like chromatin had a tendency to line up or aggregate in the center of the nucleus in a few cells. The cells displayed a remarkable tendency toward uniformity of size and shape. Mitotic figures were extremely rare, and only one or two were noted in many sections (Fig. 11). There were no giant cells, palisade arrangements, or other structural features to identify the cell

other than its relation to blood vessels and septa. The primitive appearance of these cells did not permit differentiation from fibroblasts, smooth muscle cells, or endothelial cells. However, the silver stain revealed abundant reticulum fibers forming a delicate network about each cell. This identified the cell as an undifferentiated reticular connective tissue cell (Figs. 9 and 10).

One of the important features of the tumors was the relation of the cells to the blood vessels which branched and ramified through the lobules. The delicate congeries of capillaries seemed to form the central axis about which the tumor cells were knotted and whorled (Figs. 3 and 4). This feature was most prominent in the silver stains which accentuated the delicate reticular sheath of each capillary (Fig. 9). The tumor cells were arranged concentrically outside of this sheath although they appeared to be in contact with it in most areas. The endothelial cells of the capillaries were visible occasionally and their nuclei jutted into the lumina. It would be difficult to differentiate these endothelial cells from the tumor cells except for the presence of the reticulum of the capillary wall. Apparently the capillary endothelial cells were not part of the tumor proper (Figs. 5 and 10).

There was no evidence in any of the tumors of the transition of the fusiform cells to a cell of epithelioid or lymphocytic type. There were no structures that could be identified as imperfect or immature Hassall's corpuscles, and no squamoid elements were noted. In 5 of the 7 cases portions of the partially involuted thymus were present outside the capsule of the tumor. These were composed of Hassall's corpuscles, thymic epithelial cells, and lymphocytes. This was not noted about the capsules of the other two tumors although it was assumed that further blocks of the attached areolar tissues would reveal normal, involuted thymic elements.

There was no evidence of hemorrhage, cystic necrosis, or calcification although in a few sections hemosiderin-laden phagocytes were present. Dilated lymphatics were prominent in the capsules and fibrous septa in some areas.

In summary, these tumors were lobulated, encapsulated, superior mediastinal growths composed of spindle-shaped cells concentrically arranged about delicate central blood vessels. Lymphocytes and thymic epithelial cells were present in varying numbers, suggesting portions of thymic cortex which were being replaced by tumor. The spindle-shaped cells embedded in a network of argyrophil fibers were identified as modified reticular connective tissue cells of mesenchymal origin.

Discussion

The assumption that these tumors arise in the thymus gland is supported by a number of factors. Firstly, they are all well encapsulated, midline, superior mediastinal tumors loosely adherent to the basal pericardium and great vessels. In no instance is there invasion of the overlying sternum, and origin from lymph nodes or other mediastinal structures cannot be demonstrated. As pointed out by Crosby,[2] Foot,[15] and Margolis,[3] these are factors indicating thymic origin of a tumor. Secondly, the presence of normal thymic lobules including Hassall's corpuscles in the capsules of five of the cases is strong evidence indicating an intimate relationship of the tumors to the thymus. Thirdly, the presence of masses of lymphocytes is an invariable feature of these tumors and clearly suggests a relationship to the thymus. That lymphocytes should be present in a tumor of thymic origin is not surprising in view of the lymphocytic nature of this organ.[16-18] It is recognized that these small round cells could be interpreted as specific histologic elements of the thymus gland and, as such, might be differentiated from lymphocytes.[3,19,20] Danchakoff[21] and more recent authors[11,13,15,16] were unable to make this differentiation and considered these cells to be lymphocytes. Likewise, such a differentiation could not be made in the case of these tumors. Nevertheless, it is believed justifiable to construe the presence of such cells as an indication of the thymic origin of the tumors.

In attempting to trace the cellular origin of many tumors it has been the practice to utilize embryologic material to demonstrate the origin. This was attempted with respect to the tumors described in this paper. A number of fetal thymic glands were studied in an effort to demonstrate a cell which might be considered as giving origin to the tumors. In this study of fetal thymic glands no clue was obtained as to the cell of origin although each germ layer was carefully considered.

The endodermal epithelium which forms the thymic epithelial reticular cells and the ectodermal epithelium from the cervical sinus which forms the Hassall's corpuscles were thought to represent the tissues from which these tumors might be derived. The literature strongly emphasizes the epithelial nature of the vast majority of thymic tumors. In fact, Foot[15] stated that "One must be on the alert, however, for types of thymic tumor that resemble the large spindle cell fibrosarcoma but are, in reality, made up of the entodermal reticulum cells and are therefore of epithelial nature." Lowenhaupt[9] classified two of her tumors as thymomas of epithelial origin (her group V) although she expressed some doubt and stated that the embryologic

relations were not clear. These two tumors are considered identical with the seven described here. In addition, Leyton, Turnbull, and Bratton,[22] Symmers and Vance,[4] Poer,[10] and Miller[23] (second case) also described tumors more or less resembling these. Most of these authors considered their tumors to be of epithelial origin. The case reported by Poer demonstrated the difficulty of identifying the epithelial or non-epithelial nature of some thymic tumors. However, in the cases described in this study the lack of epithelial characteristics, the demonstrated relation of the tumor cells to blood vessels, and the inability to show a relation to embryologic development were reasons for considering these tumors as non-epithelial. The marked differences demonstrated by silver stain between the normal thymic epithelium and the tumor cells make untenable any hypothesis as to the epithelial origin of the latter (Figs. 11 and 16).

The mesodermal elements which form the supporting framework of the thymus and possibly give rise to the thymic lymphocytes remain as the tissue of origin for these tumors. Mandlebaum and Celler[12] and Murray and McDonald[13] considered the possible connective tissue origin of their tumors. The latter authors remarked on the resemblance to hemangio-endothelioma, while Symmers and Vance,[4] Foot,[15] and Anderson and Conrick[8] were satisfied to call some thymic tumors "peritheliomas."

Further evidence of the mesenchymal origin of these tumors is obtained through the study of fully developed thymus glands. It was believed that the cell of origin might well be related to the mechanisms of involution of the thymus rather than to its embryologic evolution. In glands removed from persons between the ages of 10 and 30 years, the usual pattern of involution was noted as described in detail by Hammar[16] and others, *i.e.*, an increase in the width of fibrous septa at the expense of cortex, and infiltration with adipose tissue. Additionally, at the periphery of the normal lobule, subjacent to the septa or capsule, a varying number of fusiform cells were found which were identical to the tumor cells (Figs. 12 and 13). These cells were not abundant and were noted only in a very few areas where the fibrous septum was in close contact with a lobule and was replacing the thymic cells as part of the process of involution. They were associated with delicate reticulum fibers, as were the tumor cells, and this distinguished them specifically from the epithelial cells of the thymus (Fig. 14). They also were seen surrounding small arterioles and capillaries within the thymic lobule itself (Fig. 15). This was remarkably similar to the growth pattern noted in the tumors (*cf.* Figs. 4 and 8 with Figs. 12 and 13).

Strandberg[24] was the first to describe these cells in the involuting thymus. Hammar,[16] after outlining the various stages of age involution, stated:

"By the side of these changes and apparently even before the age involution there occurs, as Strandberg (1917) has shown, even from birth, a growing-in of elements of connective tissue into the parenchyma. This process starts especially from the adventitia of the vessels situated at the boundary of the medulla and cortex and has a progredient character, so that with increasing age a more and more abundant system of fine *circummedullary connective-tissue* bundles arises."

The architectural arrangement and the histologic details of these cells are such that, under neoplastic stimuli, they could conceivably produce tumors like those described. A certain degree of admixture with thymic epithelial cells would not be surprising.

Thus, the evidence for the thymic origin of these tumors rests on the gross location and on the histologic structure which seems to be reflected by certain mesodermal cells in the involuting thymus that are striking in their similarity to the tumor cells.

The demonstration that the cells are derived from the reticular connective tissue of the septa and from the adventitia of the blood vessels suggests the type of tumor presented by Stout and Murray[25] as the hemangiopericytoma. In 1942 these authors were able to separate what they called hemangiopericytoma from other types of blood vessel tumors, and more recently Stout[26] has described twenty-five such tumors. In the photographs and descriptions, his tumors closely resemble those presented in this paper. In fact Stout's case 10, being an anterior mediastinal tumor, seems identical. However, no mention of lymphocytes is made. The so-called hemangiopericytoma occurs in various portions of the body and apparently can arise wherever capillaries are present. The pericyte, as first described by Eberth[27] in 1871 as a specialized perivascular cell and described in greater detail by Zimmermann[28] in 1923, is thought to be the cell of origin of these tumors. These cells, according to Stout, have the ability to form collagenous fibers as well as smooth muscle elements which may cause them to appear epithelioid, as in glomus tumors. Although collagenous fibers are present in the tumors described here, no smooth muscle or epithelioid elements can be recognized. Since most histologists are not prepared to recognize the pericyte as different from the ordinary connective tissue cell of the adventitia (reticular connective tissue cell) it is, perhaps, best not to classify the tumors as "pericytomas," although the resemblance to those described by Stout is strong.

The descriptive term reticular perithelioma serves to emphasize the characteristic perivascular distribution of the cells as well as their derivation from reticular connective tissue.

Since these tumors arise from reticular connective tissue cells they may be found in a variety of places in the body. In fact, Stout's cases[26] show a number of sites of origin and Eggston and Wolff[29] described similar tumors in the nasopharynx. However, their origin in the thymus is characterized by the presence of masses of lymphocytes and can be connected with cellular changes of the process of involution. Thus, they may be considered as a separate group.

The reticular perithelioma of the thymus must be distinguished from tumors originating from cells of the thymic epithelial reticulum. These latter cells have irregular, stellate outlines, often deeply eosinophilic cytoplasm, and a round or slightly oval nucleus which contains scant chromatin (Fig. 16). Tumors derived from these epithelial cells tend to reproduce this pattern of cellular structure and to form true Hassall's corpuscles[3-5,11,15,30] in varying numbers. This pattern is not observed in any of the reticular peritheliomas nor is there a tendency to form Hassall's corpuscles. Even so the distinction may not be clear without special staining methods and numerous sections from various portions of the tumor.

The broad areas of lymphocytes in the reticular peritheliomas of the thymus may cause confusion with a lymphocytic tumor of thymic origin (Fig. 7). The latter type has been well described for the thymus gland.[1,2,4,8,18] The differentiation is again made by utilizing sections from multiple blocks of the tumors, showing areas composed chiefly of cells of the reticular connective tissue type in association with blood vessels.

Hemangio-endotheliomas arising in the mediastinum must be differentiated from reticular peritheliomas since the cells of both are closely related to the blood vessels. The tightly whorled pattern of fusiform cells is not usually observed in hemangio-endotheliomas although the cells in both types have a similar appearance. Furthermore, the endothelial cells of the blood vessels in the reticular peritheliomas seem to be distinct and separate from the surrounding tumor cells (Fig. 5). Finally, abundant reticulum fibers are not produced by the more highly differentiated endothelial cells.[31]

Summary

Seven similar, benign, anterior mediastinal tumors are described and evidence presented indicating their origin from the reticular connective tissue cells of the perivascular and periseptal tissues of the involuting thymus gland. The term reticular perithelioma is suggested for these tumors since it emphasizes their most important microscopic characteristics.

Assistance in preparing this paper was obtained from William C. Barrett, Jr., Associate Professor of Anatomy, Boston University School of Medicine.

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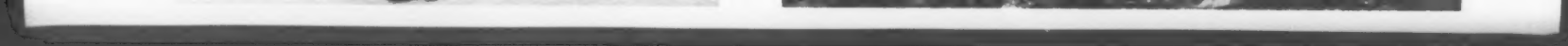
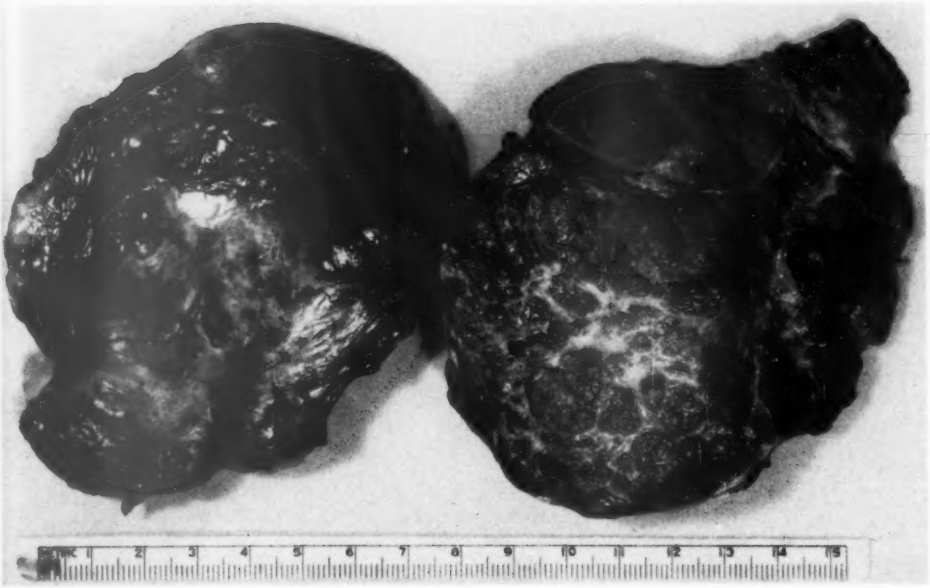
LEGENDS FOR FIGURES

FIG. 1. The cut surface of the encapsulated mediastinal tumor found at thoracotomy in case 3. The lobulated pattern and fibrous septa are shown.

FIG. 2. Thymic tumor, case 1. The spindle cells of the tumor produce an indistinct whorling pattern about numerous blood vessels. Phloxine and methylene blue stain. × 90.

FIG. 3. Thymic tumor, case 2. The tumor cells and their relation to blood vessels are well shown. Scattered lymphocytes are seen. Phloxine and methylene blue stain. × 90.





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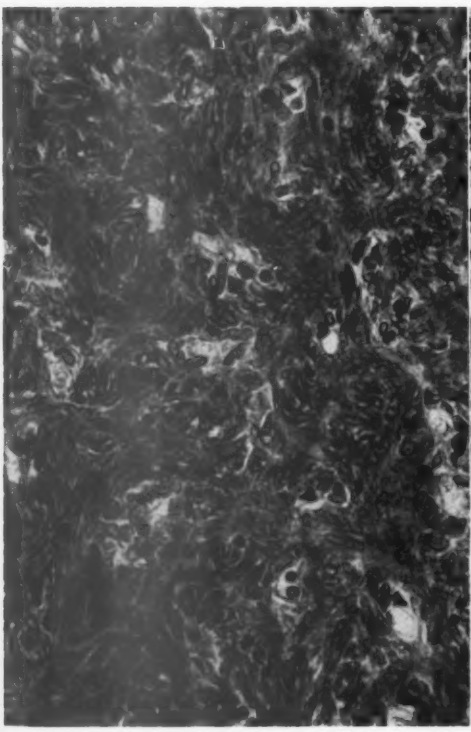

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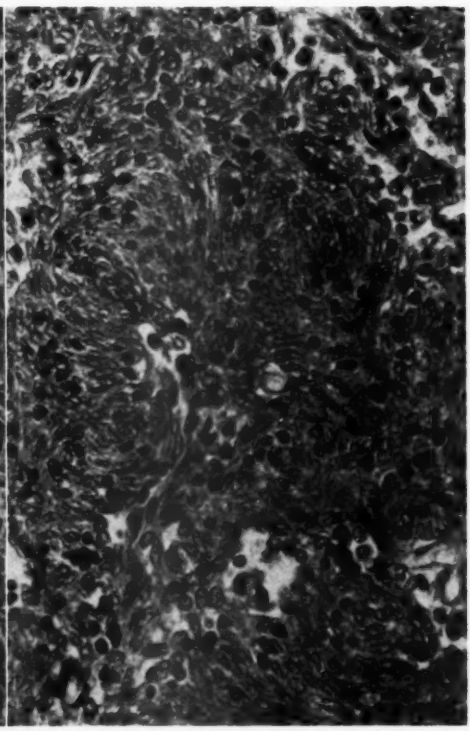

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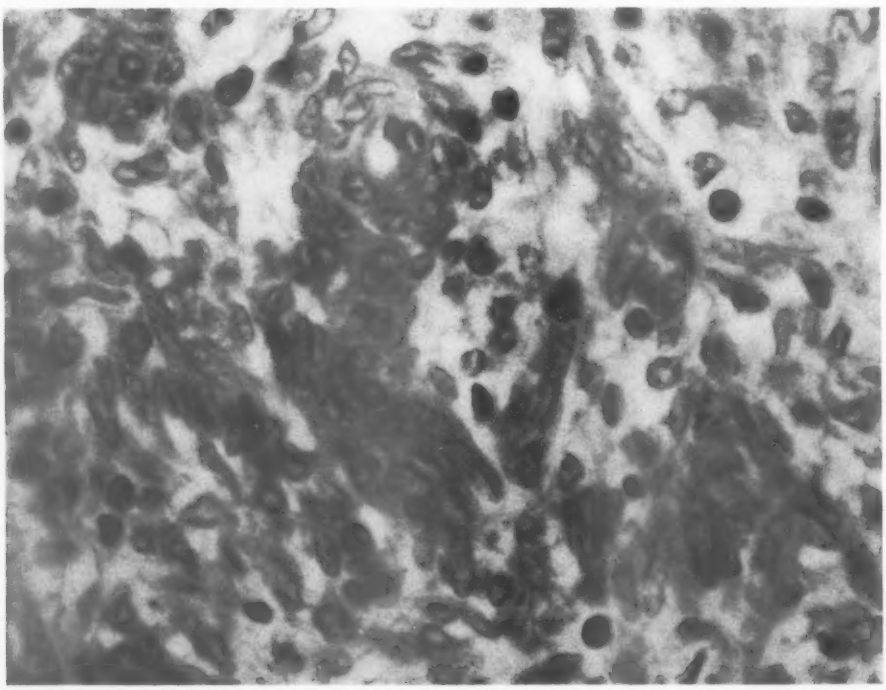
FIG. 4. Thymic tumor, case 4. The central area demonstrates the relation of the reticular cells to a blood vessel. This photomicrograph may be compared with a similar relation seen in the involuting thymus of Figure 13. Phosphotungstic acid-hematoxylin stain. × 200.

FIG. 5. Thymic tumor, case 6. The vascular pattern of the tumor is a prominent feature of this photomicrograph. The endothelial cell nuclei are readily differentiated from the tumor cells. Phosphotungstic acid-hematoxylin stain. × 140.

FIG. 6. Thymic tumor, case 5. The tumor cells spreading out from the septum and adjacent blood vessels with a dense admixture of lymphocytes are a familiar pattern in all of the tumors. For comparison with Figure 12. Phloxine and methylene blue stain. × 85.

FIG. 7. Thymic tumor, case 7. This photomicrograph was selected to show the dense accumulation of lymphocytes common to these tumors as well as the branching septae composed of tumor cells. Phloxine and methylene blue stain. × 58.

FIG. 8. Thymic tumor, case 6. The tumor cells produce a perivascular pattern similar to that seen in the involuting thymus of Figure 13. Phloxine and methylene blue stain. × 155.



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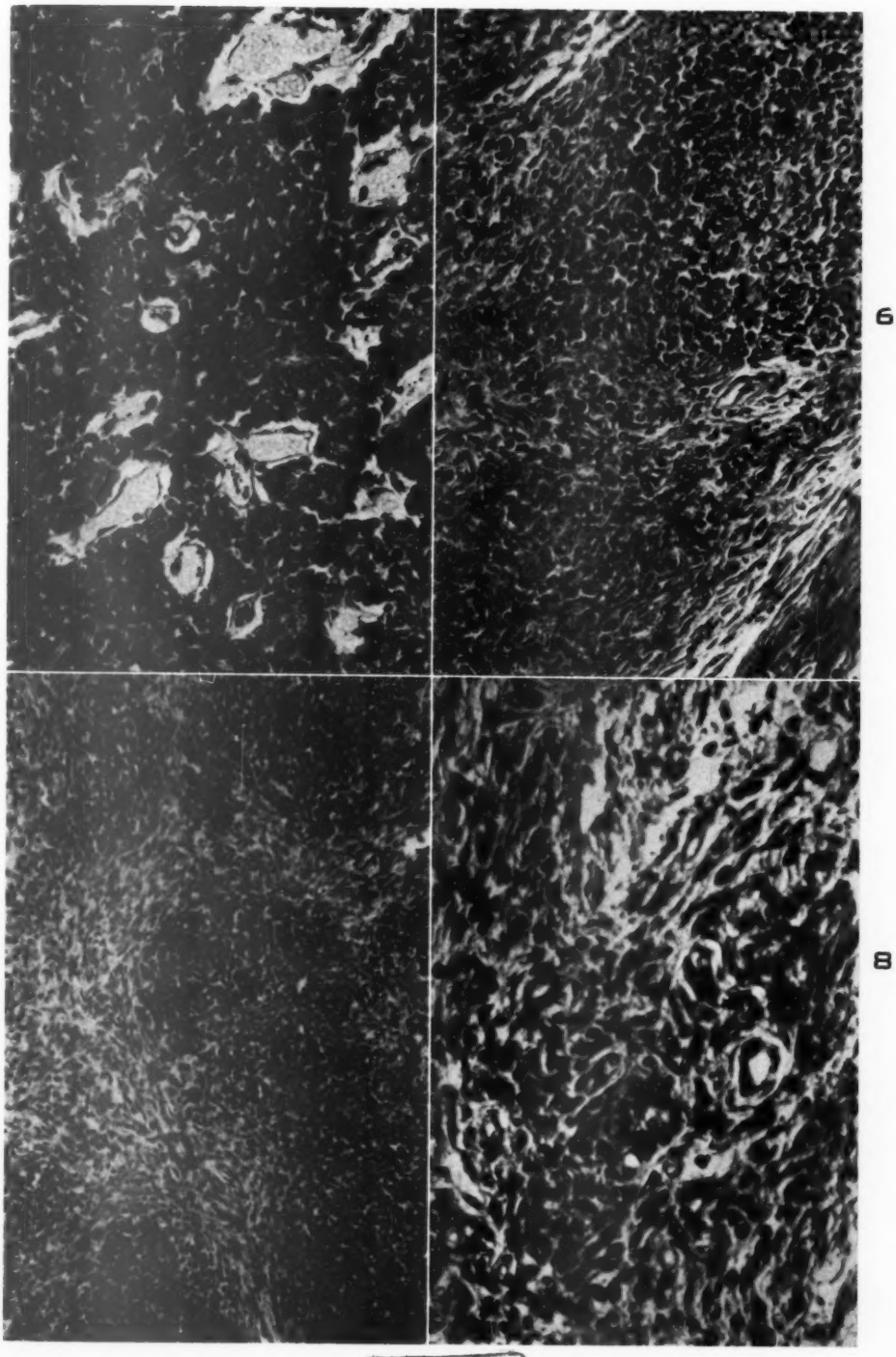

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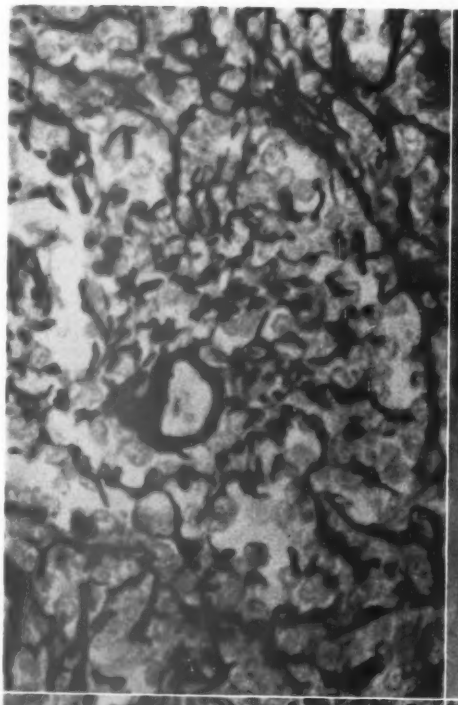
FIG. 9. Thymic tumor, case 7. Wilder's reticulum stain demonstrates the blood vessels and the dense network of fine reticulum fibers associated with the tumor cells. This pattern was noted in all tumors stained by this method. × 200.

FIG. 10. Thymic tumor, case 2. The reticulum stain brings out the sheath of the centrally located blood vessel and demonstrates the abundant reticulum associated with the tumor cells about the vessel. Wilder's silver stain, counterstained. × 270.

FIG. 11. Thymic tumor, case 3. High-power photomicrograph of the tumor cells showing their fusiform nuclei and delicate, pale, cytoplasmic processes. These cells must be distinguished from the epithelial cell of the thymus as seen in Figure 16. Phloxine and methylene blue stain. × 545.

FIG. 12. Normal involuting thymus. The interlobular septum running vertically through the center of the field is composed of reticular connective tissue cells. These cells closely resemble the tumor cells in both structure and distribution. Phloxine and methylene blue stain. × 155.

FIG. 13. Normal involuting thymus. The perivascular distribution of the reticular connective tissue cells closely resembles the pattern reproduced by the tumors, as seen in Figure 4. Phloxine and methylene blue stain. × 155.



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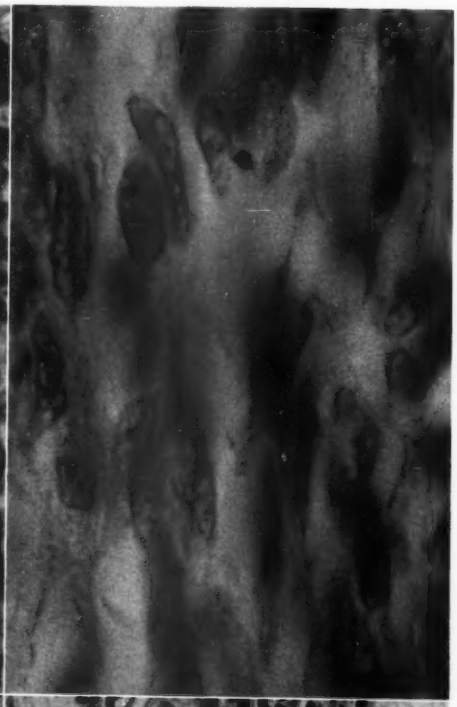

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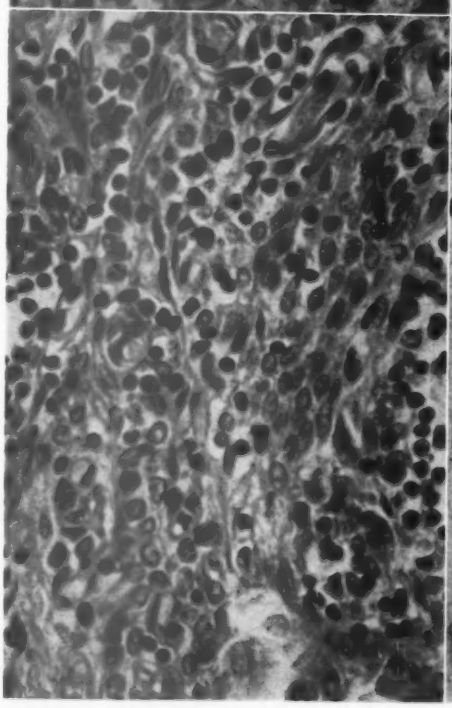

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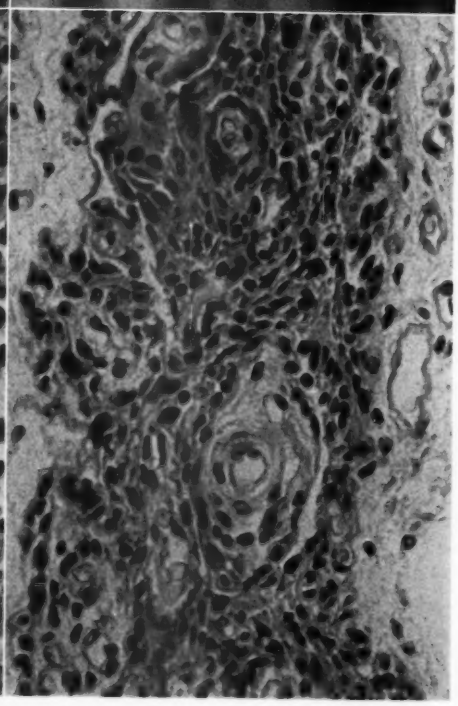

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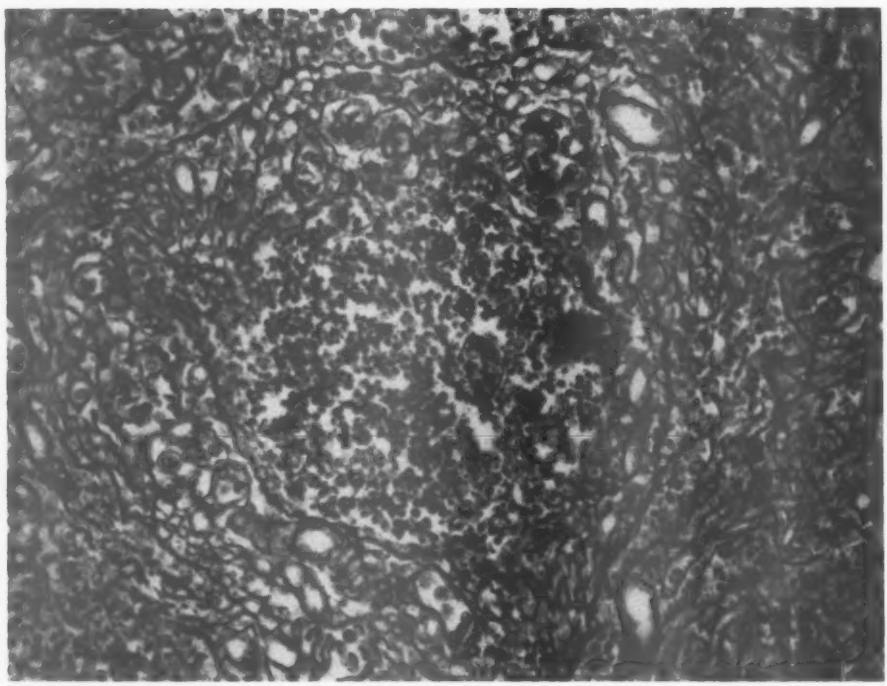
FIG. 14. Normal involuting thymus. The network of reticulin fibers has isolated a focus of lymphocytes and thymic epithelial cells as a result of the processes of involution. The reticulin fibers are associated with the perivascular and septal cells and not with the cells of the thymic lobule. Wilder's silver reticulin stain, counterstained. × 75.

FIG. 15. Normal involuting thymus. Reticular fibers are associated with the perivascular reticular connective tissue cells rather than with the cells of the thymic cortex. Wilder's reticular stain, counterstained. × 200.

FIG. 16. Normal involuting thymus. The round vesicular nuclei and rather deeply staining cytoplasm of these cells of the thymic epithelial reticulum may be compared with the spindle-shaped cells of the reticular connective tissue as shown in Figures 2 and 11. Reticulum fibers are not associated with these cells. Phloxine and methylene blue stain. × 580.



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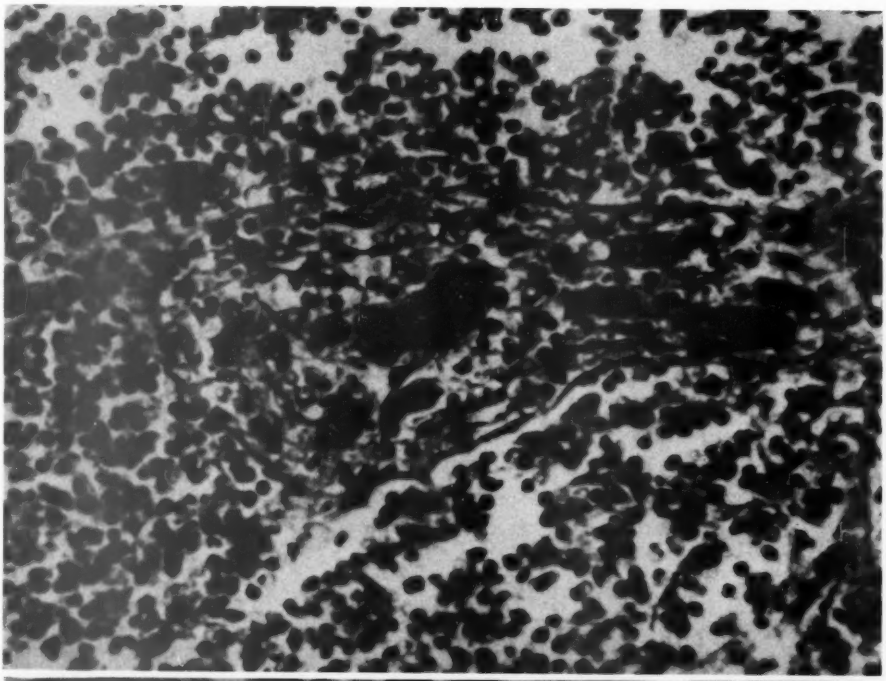

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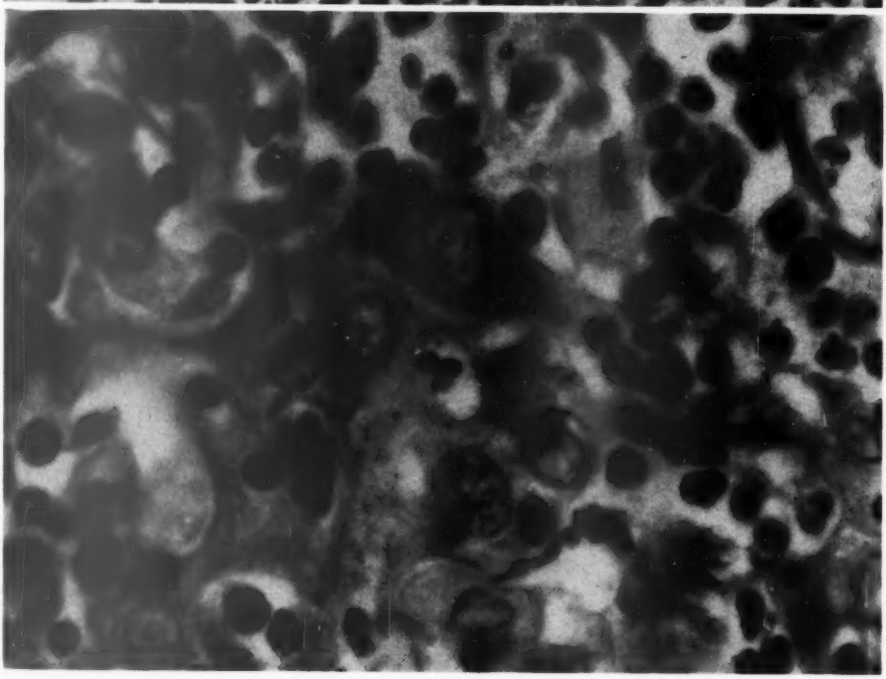

STUDIES ON THE REDUCTION OF TETRAZOLIUM DERIVATIVES BY NORMAL AND DISEASED HUMAN TISSUES *

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The enzymatic reduction of the colorless, water-soluble derivatives of tetrazolium salts to colored water-insoluble formazans has been described as differing in malignant and normal tissues. Some authors considered the differences significant enough to recommend their use for diagnostic purposes in tissue samples as well as in living persons (Straus, Cheronis, and Straus,[1] 1948; Schuermann,[2] 1950; Bunge,[3] 1951). Other authors, however (Seligman, Gofstein, and Rutenburg,[4] 1949; Masouredis *et al.*,[5] 1950), failed to confirm the differential enzymatic reduction of tetrazolium derivatives in malignant tissues of the mouse. The enzyme systems involved in the reduction of tetrazolium derivatives are not yet defined. Succinic dehydrogenase, xanthine oxidase, and also other enzyme systems might be participating (Shelton and Schneider,[6] 1952).

To evaluate the possibility of the use of tetrazolium derivatives for the diagnosis of cancer in human tissues, the reduction of triphenyltetrazolium chloride and neotetrazolium (2,2-*p*-diphenylene bis-3,5-diphenyltetrazolium chloride) in over 100 operative specimens was investigated. Neotetrazolium was found more suitable as the large crystals formed by triphenyltetrazolium chloride made the localization in the cell impossible. As a rule only a few minutes elapsed between the removal of the tissue and its incubation with tetrazolium derivatives. Slices thicker than 0.2 to 0.3 mm. were not stained uniformly. All tissues were incubated in Tyrode solution at pH 7.4 for 1 hour at 38° C., after which frozen sections were prepared. The final concentration of tetrazolium derivatives was 0.25 per cent. Triphenyltetrazolium chloride and neotetrazolium behaved in a similar fashion and are discussed together.

Normal connective tissues did not reduce tetrazolium derivatives. Fatty tissues stained pink, but since reduced tetrazolium derivative is soluble in fat it could not be decided whether the staining is due to enzymatic activity in this tissue. Striated and smooth muscles were weakly active. Endothelium of vessels were very weakly stained or remained unstained. Epithelial cells always showed reductive activity, glandular epithelium being more active than squamous epithelium.

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Liver and kidney showed strong activity. The distribution in the liver cells was uniform. In the kidney the glomeruli were negative. The proximal convoluted tubules showed the strongest activity. The collecting tubules were weakly stained.

In benign tumors the participating tissues behaved like corresponding normal tissue, *i.e.*, connective tissue was inactive, epithelium reduced tetrazolium derivatives. Chronic inflammatory tissue showed a similar behavior. Epithelioid cells in tubercles and in sarcoidosis reduced tetrazolium derivatives.

Epithelial cells in carcinomas showed reductive activity, sometimes weaker, sometimes stronger than the surrounding normal epithelium or epithelium of benign tumors. Sarcomas and the stroma of carcinomas repeatedly but not always reduced tetrazolium derivatives. No other connective tissue showed a reductive activity. In order to compare the stroma of carcinomas with the stroma of other rapidly growing tissue, the reduction of placental tissue was investigated. The stroma of chorionic villi was negative; the Langhans cells showed strong reduction. The decidual cells were weakly positive.

Stromal reduction in mammary cancer of the mouse was described by Black *et al.*,[7,8] 1951. Due to the infiltration of the tumor cells into the surrounding tissue and the staining of the stroma, malignant tumors appeared to the naked eye more diffusely stained than benign tumors. Figure 1 shows a fibro-adenoma and a carcinoma of the breast to illustrate this point. The reductive activity of metastases in the spleen was more intensive than that of the surrounding splenic tissue. In the liver, the staining of the same tumor was less intensive than that of the surrounding liver tissue. This difference is explained by the fact that the normal spleen shows a low reductive activity while normal liver tissue shows a high reductive activity. The reductive activity of the tumor investigated falls between those of these tissues. Figures 1 to 6 demonstrate the histologic appearance of inflammatory, benign, and malignant tissues.

In order to compare the findings, the increasing intensity of tetrazolium derivative reduction has been designated from minus to 4 plus. Table I shows the intensity of tetrazolium derivative reduction in inflammatory tissues, Table II that in benign, and Table III, in malignant tumors. The largest group of all tissues are those which show a reductase intensity of 2 plus. The next largest group is with benign tumors, 1 plus; with malignant tumors, 3 plus. Carcinomas give extremely variable results; a few carcinomas show no reduction and some an extraordinarily intense reaction. No correlation between the intensity of the staining or the lack of reductive activity and the clini-

TABLE I

Reduction of Tetrazolium Derivatives in Inflammatory Tissues

Organ	Type of inflammation	Intensity of reduction					
		−	±	+	++	+++	++++
Testis	Chronic non-specific			1	1		
Pancreas	Chronic non-specific			1			
Liver	Cirrhosis				1		
Lung	Tuberculosis			2			
Lymph node	Sarcoidosis				1	1	
Leg	Gangrene						
Stomach	Ulcer						
Total (8)		0	0	4	3	1	0

TABLE II

Reduction of Tetrazolium Derivatives in Benign Tumors

Organ	Diagnosis	Intensity of reduction					
		−	±	+	++	+++	++++
Breast	Fibro-adenoma		1	9	13	9	
Breast	Cystic mastopathy		1	3	8	6	
Breast	Gynecomastia				1		
Uterus	Leiomyoma		1	1			
Cervix	Squamous hyperplasia		1				
Ovary	Cyst			1			
Prostate	Benign hypertrophy			3	10	1	
Choroid plexus	Papilloma				1		
Chest wall	Cyst			1			
Mediastinum	Neurilemmoma			2			
Labium	Lipoma			1			
Ligament	Leiomyoma				1		
Total (75)		0	4	21	34	16	0

TABLE III

Reduction of Tetrazolium Derivatives in Malignant Neoplasms

Organ	Intensity of reduction					
	−	±	+	++	+++	++++
Breast	1		3	7	4	1
Cervix			1			
Ovary			1	3		
Penis				1		
Mouth				1		
Stomach				2		
Intestine	1			2	1	1
Brain			1		1	
Adrenal gland		1				
Lymph node				3	1	
Liver				1		
Lung		1		2	1	
Bladder				3	2	
Prostate				2		
Total (49)	2	2	6	27	10	2

cal malignancy of the tumor has been found. It seemed, however, that stromal reduction was associated with a high degree of malignancy. In spite of the differences in the reductive activity in malignant and benign tumors, the general agreement between them is so great that it is unlikely that the method could be used for the diagnosis of cancer in operative material.

SUMMARY

The reduction of the tetrazolium dyes, triphenyltetrazolium chloride and neotetrazolium, was investigated in biopsy material in order to establish the possible use of this technique in the diagnosis of cancer in human material.

The epithelial cells of carcinomas show generally no differential behavior from normal epithelium, or from epithelium of benign tumors.

The stroma of malignant tumors in a number of cases reduced tetrazolium derivatives. Reductive activity was not observed in any normal connective tissue.

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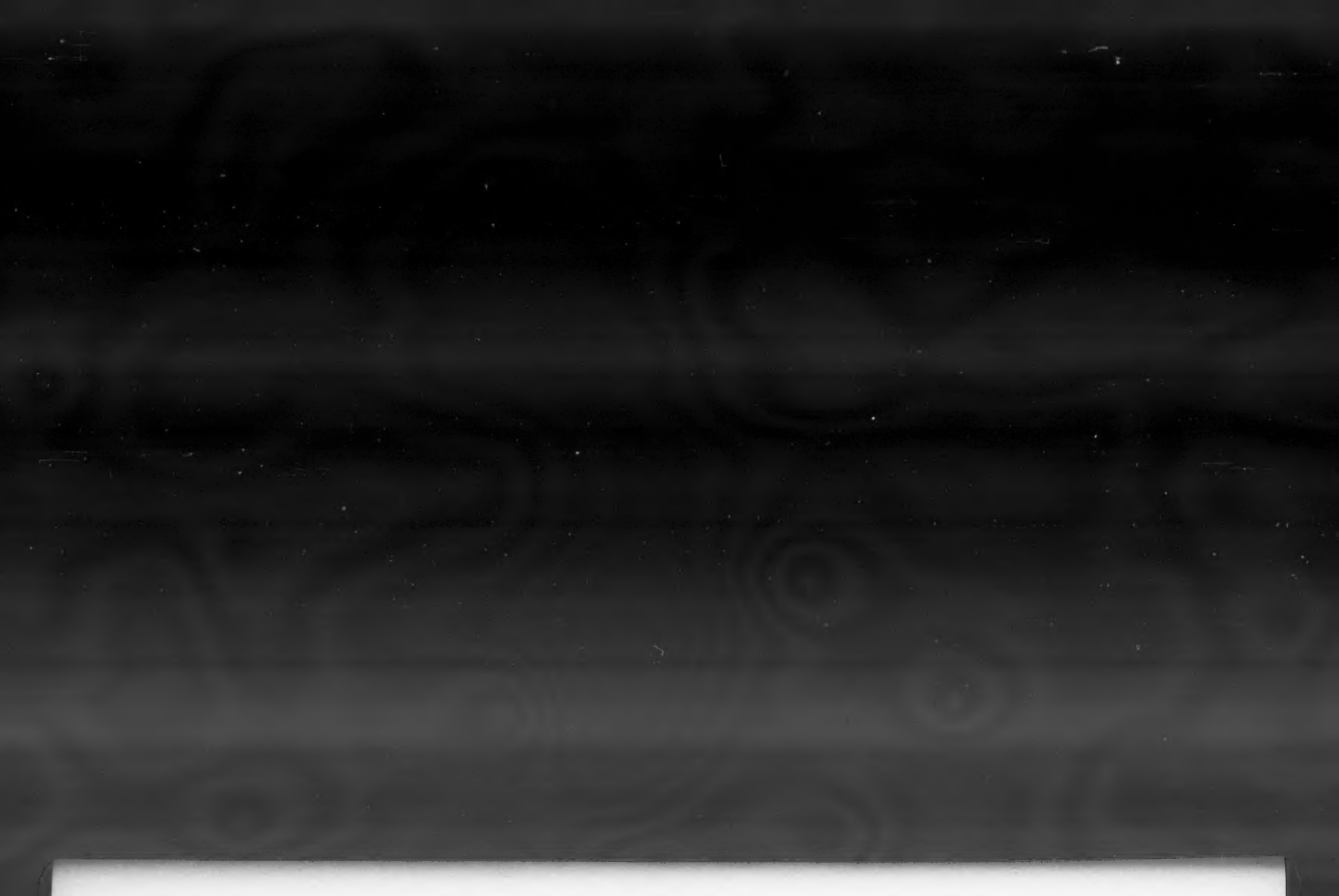
LEGENDS FOR FIGURES

All tissues have been incubated with tetrazolium derivatives. The red or violet areas of the reduced tetrazolium derivatives of the original section are black in the reproduction.

FIG. 1. Right: Fibro-adenoma of the breast. Left: Carcinoma of the breast. Two-thirds of the original size.

FIG. 2. Sarcoidosis. Reduced triphenyltetrazolium chloride in the epithelioid cells. × 150.

FIG. 3. Fibro-adenoma of the breast. Reduced tetrazolium derivatives in the epithelial lining of the acini. × 150.



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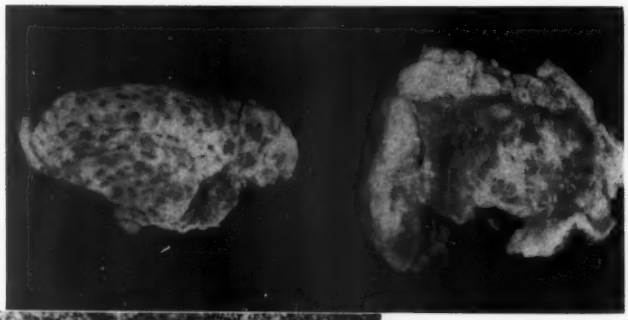

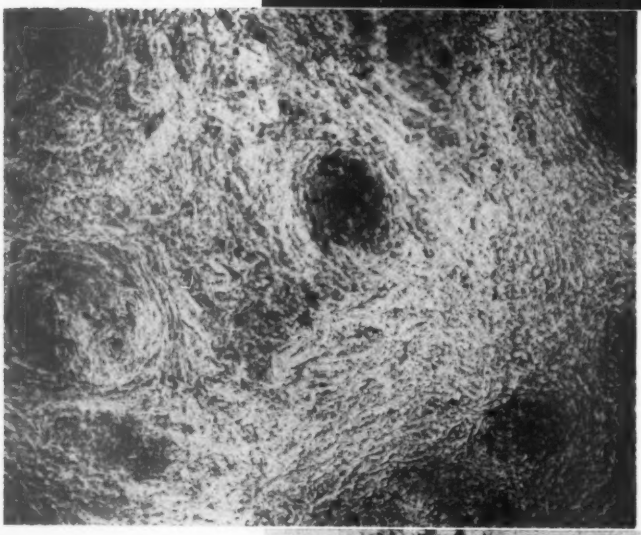

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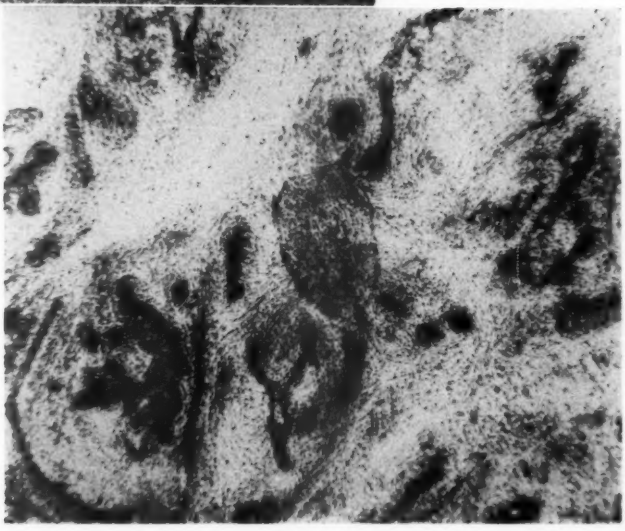
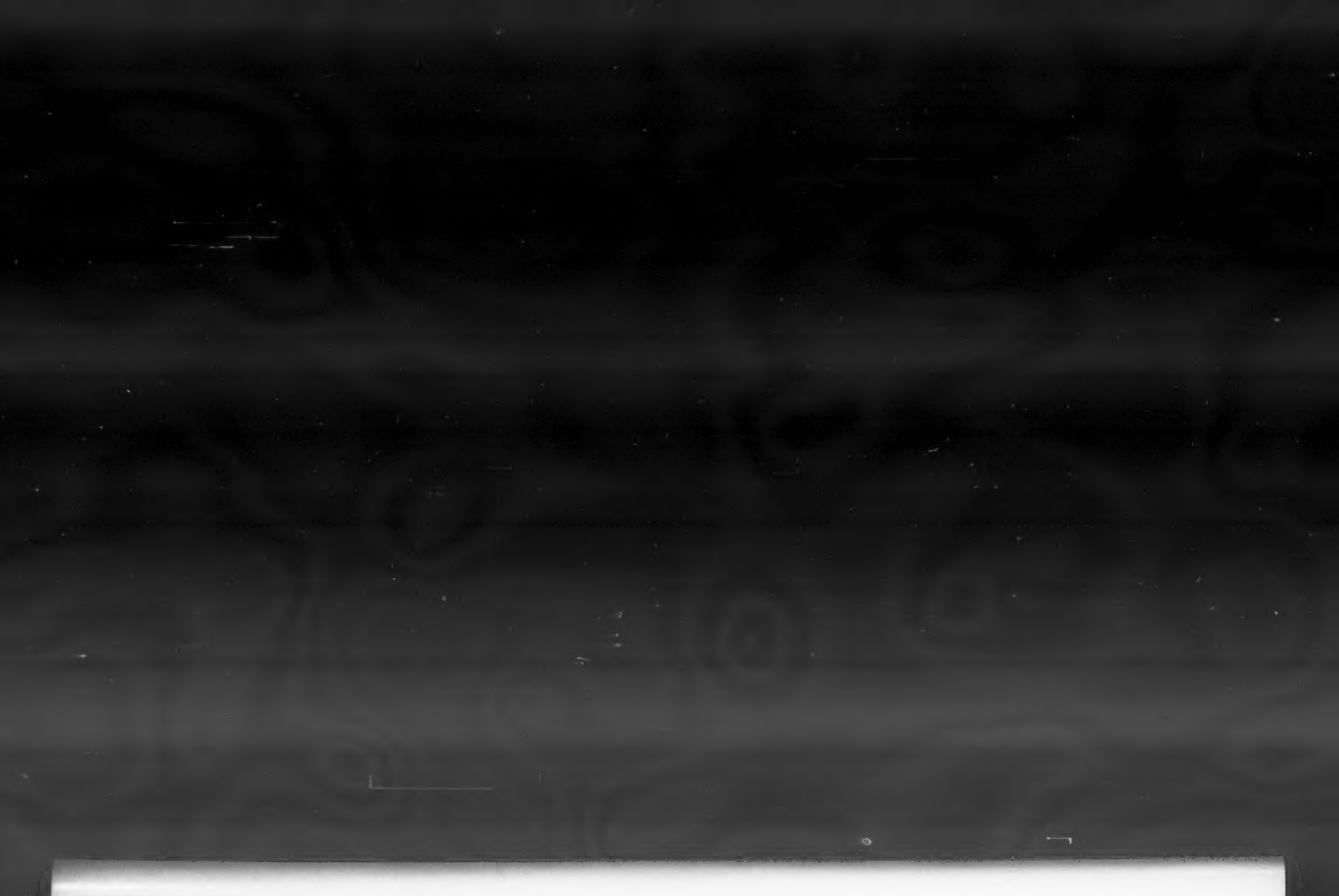


FIG. 4. Carcinoma of the breast. Reduced tetrazolium derivatives in the epithelial cells diffusely invading the stroma. × 150.

FIG. 5. Papilloma of the choroid plexus. Reduced tetrazolium derivatives in epithelial cells. × 180.

FIG. 6. Carcinoma of the colon. Reduced tetrazolium derivatives in epithelial cells. × 180.



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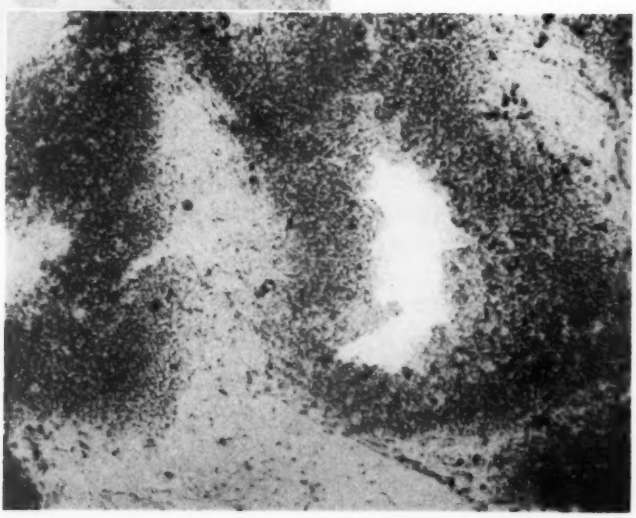

6

THE UTILIZATION OF A PURE STRAIN OF MAMMALIAN CELLS (EARLE) FOR THE CULTIVATION OF VIRUSES IN VITRO

I. Multiplication of Pseudorabies and Herpes Simplex Viruses *

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Relationships between viruses and cells have been studied by the employment of bacterial viruses and bacterial cells. This phage-bacteria system has made possible the study of the phenomena of viral adsorption and penetration, altered cellular metabolism following viral parasitism, and the investigation of the genetic, metabolic, quantitative, and temporal aspects of viral reproduction. For mammalian viruses, however, no analogue of the phage-bacterial system has existed.

Cells ideally adaptable for the study of mammalian virus-like relationships were made available by Earle and his co-workers.[1-10] These investigators developed a "pure strain" of mouse cells, designated as the L strain. Subcutaneous tissue from a C_3H mouse was the source of a strain of fibroblasts which were treated with 20-methylcholanthrene and subsequently acquired the capacity to produce sarcomas following inoculation into C_3H mice.[1-3] In 1948, the cultivation by Sanford, Earle, and Likely[4] of a single cell from this strain resulted in a strain of cells which have a defined heritage, *i.e.*, a "pure cell strain." The morphology of these cells in culture has been described by Earle *et al.*[1,5,6] Although the cells were originally grown in a plasma coagulum, they have since been modified in Earle's laboratory to grow without clotted plasma on cellophane[7] or on glass.[8] Techniques are available for the preparation of replicate cultures of these cells in large numbers[9] and for the measurement of cellular populations by the enumeration of cellular nuclei.[10] Recognition that the growth of a mammalian virus in cells from this pure strain would provide a system which closely resembles the bacteriophage-bacterial system led to the present series of investigative studies.

The purpose of this communication is to report the findings of studies which show that the viruses of pseudorabies and herpes simplex multiply in L strain cells. These experimental studies include observa-

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A preliminary report of these findings was presented at the annual meeting of the Society of American Bacteriologists, Boston, April 28, 1952 (*Bact. Proc.*, 1952, pp. 82–83).

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tions upon the relationship of the physiologic activity of the cells to viral multiplication.

Materials and Methods

Viruses. Pseudorabies virus, Aujesky strain, was obtained from Dr. R. E. Shope through the facilities of the American Type Culture Collection. It was received as infected rabbit brain preserved in glycerol. Subsequently, three passages were performed by intracranial transfer in Swiss albino mice, CFW strain. Brain tissue representative of the third passage in mice was triturated by mortar and pestle in 5 per cent dextrose in distilled water to yield a 10 per cent suspension. Following centrifugation at 1500 r.p.m. for 10 minutes, the supernatant fluid was stored in 2 ml. glass ampoules * at the temperature of dry ice. The intracranial inoculation of this supernatant fluid into mice established the LD_{50} as $10^{-3.8}$ per 0.03 to 0.05 ml.

Herpes simplex virus was isolated from a patient in 1946 by Dr. J. T. Syverton. It was preserved following a single transfer, as lyophilized mouse brain suspension until 1951 when five intracranial passages in mice were carried out. Ten per cent brain suspensions were prepared and stored as has been outlined for pseudorabies virus. Mouse titration after five passages revealed the LD_{50} to be $10^{-6.0}$ per 0.03 to 0.05 ml. of 10 per cent suspension.

Assays for Viruses. Quantitative measurements of both pseudorabies and herpes simplex viruses were made by intracranial inoculation, 0.03 to 0.05 ml., of serial tenfold dilutions of viral material into groups of 4 or 5 Swiss albino mice, CFW strain, 3 to 8 weeks of age. Hanks' balanced salt solution was used as the diluent. The mice were observed daily for 2 weeks for objective evidence of illness or death resulting from these viruses. The LD_{50} was calculated by the Reed and Muench method.[11]

Cells. The L strain of mouse cells was kindly supplied by Dr. W. R. Earle in June, 1951.

Media for Cellular Cultures. Three cultural media were utilized: a horse serum-embryonic extract mixture; 0x serum ultrafiltrate† (1 part) diluted with Hanks' salt solution (2 parts); a maintenance solution.

The first medium was composed of horse serum, 40 per cent; chicken embryonic extract (1:1), 20 per cent; and Hanks' balanced salt solution, 40 per cent; it is designated as HoS-40, EE-20, H-40. Sterile horse serum was obtained from normal horses by jugular venipuncture. Approximately 2000 ml. of blood were collected from each horse in a 6000 ml. Erlenmeyer flask. The clotted blood was stored at 4 to 6° C. for 24 to 48 hours, and the serum was then withdrawn into either sterile 25 ml. sealed glass ampoules* or screw cap test tubes, 16 by 150 mm., for storage at −20° C. No inactivation of complement by heat was carried out.

Chicken embryonic extract was prepared from 9-day embryos by the syringe method[12] modified by the employment of one instead of two stainless steel wire screens, 28 mesh. A volume of Hanks' solution was mixed with an equal volume of crushed embryo. Following centrifugation at 2500 r.p.m. for 20 minutes, the supernatant 1:1 extract was stored in 1 ml. aliquots at −20° C. Before use, it was thawed, centrifuged at 2500 r.p.m. for 20 minutes, and only the supernatant liquid was employed.

The second cultural medium was prepared by diluting 1 part of 0x serum ultrafiltrate with 2 parts of Hanks' solution. This will be designated UF-33, H-67.

The third medium was prepared by the slight modification of a formula for a

* Kimble Neutraglas, Toledo 1, Ohio.

† Microbiological Associates, Bethesda, Md.

maintenance solution supplied by Dr. J. H. Hanks and was employed as the undiluted mixture (MS-100).*

* This solution provides materials for the maintenance of cells *in vitro*, but is inadequate for cellular growth. The balanced salt solution is that of Hanks and Wallace[23] with increased glucose. The organic constituents are essentially similar to the solution of Anfinsen *et al.*[24] as modified by Hanks.[25]

Components of maintenance solution	*Final concentration in mg./liter*
I. Hanks' balanced salt solution	
$CaCl_2$	140
KCl	400
NaCl	8000
$Na_2HPO_4 \cdot 2H_2O$	60
$NaHCO_3$	1000
$MgSO_4 \cdot 7H_2O$	200
KH_2PO_4	60
Glucose	2000
II. Amino acids	
Parenamine 15% (Winthrop-Stearns, Inc., New York 13, N.Y. Approximate analysis in percental amounts: total nitrogen, 2; total alpha amino nitrogen, 1.4; total ash, 0.2; approximate pH, 5.6; lysine, 1.25; valine, 1.10; leucine, 1.60; arginine, 0.60; histidine, 0.25; threonine, 0.30; isoleucine, 1.00; methionine, 0.50; tryptophane, 0.15; phenylalanine, 0.50.)	300
Glycine	20
Histidine	20
Cystine	15
Glycerol	500
Na acetate	500
Na pyruvate	500
Succinic acid	10
l-malic acid	5
Para-aminobenzoic acid	0.01
Thiamine	1.0
Nicotinamide	0.5
Co-carboxylase	0.5
Ca-pantothenate	0.5
Pyridoxal	0.5
d-ribose	0.5
Riboflavin	0.5
Choline	1.5
Biotin	0.01
Inositol	1.5
Folic acid	0.01
Xanthine	1.0
Guanine	1.0
Thymine	0.5
Adenine	2.5
Uracil	1.0
Cytosine	0.5
III. Phenol red	20

The maintenance solution was prepared from a stock solution which contained 10 times

Sterility of media was determined by culture in liquid thioglycollate at 37° C. and room temperature.

Techniques for Cellular Cultivation. L strain cells were cultured in Porter flasks. Cells were scraped from the glass wall with a bent tip glass capillary pipette and suspended in 0.2 to 0.5 ml. of the medium overlying the cells. An aliquot, 1 or 2 drops, of this suspension was placed on the flat wall of a clean sterile Porter flask. After 10 to 15 minutes at room temperature to allow time for adhesion of the cells to the glass, fresh medium (HoS-40, EE-20, H-40), 1 ml., was added to each flask. Incubation was carried out at 36 to 37° C. with the flasks in a stationary horizontal position. At intervals of from 2 to 5 days, either one-half or all of the liquid medium was removed and replaced with an equal volume of fresh medium. Within 7 to 10 days, when cells covered the bottom surface of the flask, the cultures were inoculated with virus. Microscopic visualization of the cells was performed during cultivation by the utilization of a microscope with a 10x (16 mm.) objective and 10x ocular. When cytologic examination of cells was desired at a higher magnification, the cells that had grown on a coverglass, 11 by 22 mm., in a Porter flask were stained with hematoxylin and eosin.

Experimental

The potentialities of L strain cells to support the multiplication of viruses were studied with three objectives in mind: (1) To establish unequivocally viral multiplication in these cells. (2) To study the behavior of certain murine and non-murine viruses in cultures of these cells. Neurotropic murine viruses were employed initially because of their availability in this laboratory. The results with the viruses of pseudorabies and herpes simplex are reported here. Studies are now in progress with type 2 poliomyelitis, MM, mouse encephalomyelitis, Coxsackie, Eastern equine encephalomyelitis, lymphocytic choriomeningitis, and vaccinia viruses. (3) To determine the relationship of cellular physiologic activity to the multiplication of virus in L strain cells. Accordingly, virus was cultivated in cells kept at three levels of metabolic activity by the employment of three cultural environments: (a) A medium composed of 40 per cent horse serum, 10 per cent chicken embryonic extract (1:1), and 50 per cent Hanks' salt solution (HoS-40, EE-10, H-50) at 37° C. for experiments 1 and 6. Cells in this environment were actively metabolizing and proliferating. (b) A mixture of ox serum ultrafiltrate and Hanks' solution, UF-33, H-67, at 37° C. (experiments 3 and 8). Cells which were maintained in this

the final concentration of ingredients for block I, and from stock solutions, 100 times final concentration, for blocks II and III. Several drops of chloroform were added as preservative and the stock solutions stored either at room temperature or 4 to 6° C. Sterilization was performed by autoclaving (10 lbs., 10 minutes) the mixture, excluding the vitamins, in 20 ml. lots in pyrex bottles. To avoid the reduction of glucose by amino acids, it was placed within the bottle in a separate glass compartment which consisted of a piece of pyrex glass tubing, closed at one end. After autoclaving, the glucose was mixed with the other solution in the bottle by inverting the bottle. The mixture of B vitamins was added after sterilization by passage through a Selas 0.03 porosity filter. The resultant solution, after adjustment to an initial pH of 7.4 to 7.6 with 1.5 ml. of sterile 1.4 per cent sodium bicarbonate solution, was the complete maintenance solution, 100 per cent (MS-100).

manner multiplied and metabolized slowly. (c) A maintenance solution (MS-100), at 22 to 25° C. for experiments 4 and 9. Cellular metabolic activity in this environment was markedly reduced.

The propagation of L strain cells is customarily carried out in a nutritive medium composed of HoS-40, EE-20, H-40.[4,8] In this medium at 37° C., there is active cellular proliferation and metabolism. When, for these experiments, it was desired to maintain cells for 14 to 20 days, it was found necessary to reduce the quantity of embryonic extract employed after the initial period (7 to 10 days) of cellular cultivation. Otherwise, excessive cellular proliferation frequently resulted in overcrowding of cells in the flasks. A medium composed of horse serum, 40 per cent, with 0 or 10 per cent embryonic extract was found to maintain normal cellular structure and active cellular metabolism (as evidenced by decrements in pH) and yet to prevent excessive cellular proliferation.

OBSERVATIONS WITH PSEUDORABIES VIRUS

Six experiments were performed to determine whether cultures of L strain cells would support the multiplication of pseudorabies virus.

Cells Cultivated in Horse Serum-Embryonic Extract Medium at 37° C.

Two experiments were carried out to determine whether physiologically active L strain cells would support the growth of pseudorabies virus.

Experiment 1. Two Porter flasks which contained adequate cellular populations were employed for each experiment. The liquid medium was removed, and replaced with a mixture of viral suspension, 0.1 ml., and fresh HoS-40, EE-10, H-50, 0.9 ml. At the intervals designated by the points on the curves in Text-figure 1, aliquots of supernatant liquid, 0.25 ml., were removed from each of two flasks, pooled, tested qualitatively for virus in mice, and stored at the temperature of dry ice for subsequent quantitative assay for virus. To maintain the proper pH of the cultural liquid, sufficient sterile 1.4 per cent sodium bicarbonate solution was added whenever the pH was below 6.7 to 6.8. On the fifth or sixth day of viral cultivation, fresh HoS-40, EE-10, H-50 mixture was added to each flask, either as a supplement to bring the volume of liquid again to 1 ml. or as a 1 ml. replacement, after the complete removal of old medium.

The results of experiment 1A are presented in Table I and Text-fig. 1. Table I presents the protocol for this experiment, illustrates the method which was employed to analyze the data, and records the results. Virus was measured in the liquid phases of the cultures. Occasionally cells which had loosened from the glass were present in this liquid.

The concentration of pseudorabies virus present in the liquid phase of the cultures on the days indicated in Table I are listed as the logarithmic value and as the equivalent numerical value. To determine for one cultural flask the quantity of virus present in the total volume of liquid, and the total yield of virus during the period of

cultivation, the following calculations were made: (1) The quantity of virus present in the liquid phases of the cultures as LD_{50} was calculated by multiplying the numbers of LD_{50} per 0.05 ml. of liquid by the number of 0.05 ml. aliquots contained in the volume of liquid present. Although the volume of viral suspension which was inoculated into each mouse varied from 0.03 to 0.05 ml., the divisor for this calculation was taken as 0.05 to make the quotients and the numbers of LD_{50} present or removed minima. The quantity of virus in the fluid which was removed from the cultures was calculated similarly from the numbers of LD_{50} per 0.05 ml. and the volumes of liquid removed. (2) The number of LD_{50} produced into the liquid phase is a cumulative figure and was calculated by the following formula:

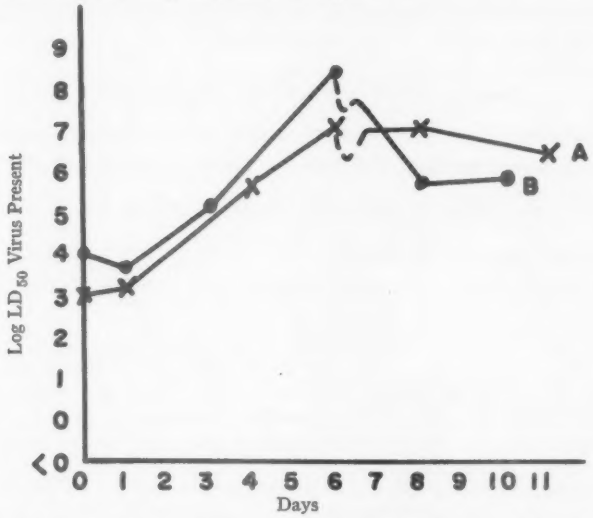
$$V^Y = V_p + V_r - V_i$$

V^Y = The yield of virus produced into the liquid phase of the culture by day of viral growth d.

V_p = The amount of virus present in the liquid phase on day d.

V_r = The amount of virus removed from the liquid phase prior to day d.

V_i = The amount of virus inoculated on day 0.



Text-fig. 1. Results for experiment 1. The growth of pseudorabies virus when measured in the liquid phase of L strain cultures which employed HoS-40, EE-10, H-50 at 37° C.

Table I makes known for experiment 1A the observation that the net yield of pseudorabies virus was more than 3×10^8 LD_{50}. Moreover, it can be seen that most of the virus was produced by the sixth day.

Text-figure 1 presents graphically for experiments 1A and 1B the growth of virus as reflected in the liquid phases of two sets of cultures, A and B. These results are shown on semi-log co-ordinates for ease of presentation. It is not intended to imply that any straight line portions of these curves signify geometrically progressive viral multiplication. The dotted line portions of the curves represent the transitory

TABLE I

Results from Experiment 1A. Method for Analysis of Data
Pseudorabies Virus

Duration of cultivation		Viral concentration in cultural liquid		Volume of liquid present	Quantity of virus present		Volume of liquid removed	Quantity of virus removed	Quantity of virus produced
Cellular	Viral	Log value	Equivalent numerical value		Numerical value	Equivalent log value			
days	*days*	*LD_{50}/0.05 ml.*	*LD_{50}/0.05 ml.*	*ml.*	*LD_{50}*	*LD_{50}*	*ml.*	*LD_{50}*	*LD_{50}*
8	0	2.8*	630	1	12,600	4.10			0
9	1	2.5	320	1	6,400	3.81	0.25	1,600	0
11	3	4.0	10,000	0.85†	170,000	5.23	0.25	50,000	159,000
14	6	7.4	25,000,000	0.65†	325,000,000	8.51	0.65	325,000,000	325,039,000
16	8	4.5	32,000	1	640,000	5.81	0.25	160,000	325,679,000
18	10	4.7	50,000	0.80†	800,000	5.90	0.80	800,000	325,999,000

* The inoculum of pseudorabies virus for this experiment was 0.1 ml. of a suspension of mouse brain tissue which contained $10^{2.8}$ LD_{50} per 0.05 ml. of suspension.

† The apparent discrepancies between the quantities of liquid which are shown to be present and the amounts which might be anticipated to exist following the removal of the indicated volumes of liquid resulted from the addition to the cultures of sufficient 1.4 per cent sodium bicarbonate solution to raise the pH of the liquid in the flasks to 6.8 to 7.2.

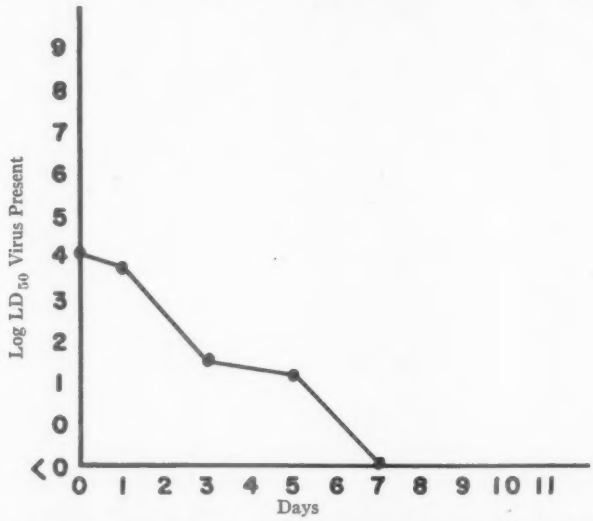
decrements in the quantity of virus present in the liquid phase of the cultures which occurred when the fluid medium was replaced.

It can be seen from the curves shown in Text-figure 1 that multiplication of pseudorabies virus occurred. Virus in the cultures increased to levels in excess of the quantities of virus present immediately following inoculation. Thus, net positive yields of virus resulted. It is obvious that such quantitative increments in virus could not have resulted from the adsorption of virus to cells and its subsequent elution into the liquid phase of the cultures, although these processes probably occurred in these cultures.

Survival of Pseudorabies Virus in Cell-Free Medium, at 37° C.

As a control for experiment 1, the persistence of virus in the absence of cells was determined.

Experiment 2. A mixture of pseudorabies virus and the non-cellular components of the medium in which normal L strain cells had been cultivated for 4 days was employed. The medium was rendered free of cells by centrifugation at 2000 r.p.m. for 15



Text-fig. 2. Results from experiment 2. The survival of pseudorabies virus in cell-free medium.

minutes. Into each of four Porter flasks were placed 0.9 ml. of cell-free fluid and 0.1 ml. of pseudorabies virus. The flasks were incubated at 36 to 37° C. At the times designated by the points on the curve shown in Text-figure 2, 0.1 ml. of liquid was removed from each flask, pooled, and stored at the temperature of solid carbon dioxide for subsequent titration for viral content in mice.

The results of experiment 2 are presented in Text-figure 2. It is evident from the curve shown that virus deteriorated rapidly during

the first 72 hours of incubation and at a slower rate during the next 2 days. By 7 days virus had disappeared completely. These findings show that virus progressively disappears in cell-free medium and support the conclusion that the survival of virus in experiment 1 beyond the seventh day resulted from the presence of cells and presumably from the multiplication of virus within cells.

Cells Cultivated in Ox Serum Ultrafiltrate Medium at 37° C.

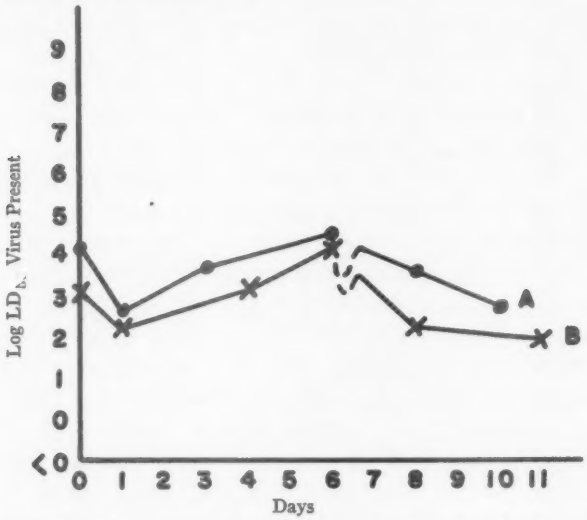
Ox serum ultrafiltrate medium was originally employed for the maintenance of L strain cells with two purposes in mind: to study the cells in an environment other than the usual horse serum-embryonic extract mixture and to maintain the cells for prolonged intervals without cellular transfer. It was found that L strain cells which were maintained in UF-33, H-67 at 37° C. for at least 10 to 12 days: multiplied slowly, if at all; were transferable consistently only after re-exposure to a horse serum-embryonic extract medium for several days prior to subculture; and were altered morphologically. To illustrate the change in structure which occurred when L strain cells were placed in UF-33, H-67, cells from cultures which employed horse serum-embryonic extract (Fig. 1) may be compared with cells from cultures which utilized ox serum ultrafiltrate medium (Fig. 2). The cells which were cultured in horse serum-embryonic extract medium consistently showed an abundant cytoplasm and an occasional large multinucleated form (Fig. 1). In contrast, cells maintained in UF-33, H-67 at 37° C. for more than 2 or 3 days lost much of their cytoplasm, although numerous intercellular cytoplasmic strands usually remained (Fig. 2).

The purpose of experiment 3 was to measure the capacity of pseudorabies virus to multiply in L strain cells under cultivation in UF-33, H-67 at 37° C. These findings were desired for comparison with the results that were obtained with the HoS-40, EE-10, H-50 medium (experiment 1).

Experiment 3. Porter flask cultures of L strain cells were employed in pairs. One or 2 days prior to viral inoculation, the HoS-40, EE-20, H-40 medium was removed and replaced by 1 ml. of UF-33, H-67. On the day of viral inoculation, the medium was removed from each flask and replaced with a mixture of fresh UF-33, H-67, 0.9 ml., and viral suspension, 0.1 ml. The preliminary period of exposure to UF-33, H-67 at 37° C. was carried out for two reasons: to eliminate as much as possible, by dilution, the HoS-40, EE-20, H-40 medium previously present in the cultures, and to permit the cells to adapt to the new environment before being exposed to virus. Measurements for virus were made in the liquid phases of the cultures at the intervals designated by the points on the curves in Text-figure 3. On the fifth or sixth day of viral cultivation the liquid medium was either removed and replaced, or supplemented to make a final volume of 1 ml.

The results of experiments 3A and 3B are shown in Text-figure 3.

It can be seen that virus multiplied in these cultures, for the quantities of virus present in the liquid phases of the cultures on the sixth day of each experiment exceeded those present immediately following inoculation. However, the extent of viral multiplication in these cultures which contained ox serum ultrafiltrate medium was significantly less than that obtained from cultures which utilized horse serum embryonic extract medium (experiment 1).



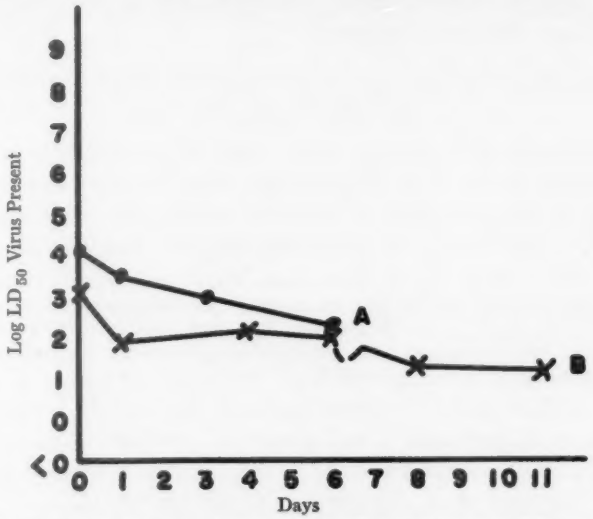
Text-fig. 3. Results from experiment 3, The growth of pseudorabies virus when measured in the liquid phase of the L strain cultures which employed UF-33, H-67 medium at 37° C.

Cells Cultivated in Maintenance Solution at 22° to 25° C.

The use of a synthetic solution permits precise definition and standardization of the initial composition of the liquid phase in which cells are cultivated. This property of a tissue culture medium is especially desirable for the study of cellular or viral metabolism. Accordingly, experiment 4 was designed to measure the production of pseudorabies virus in L strain cells maintained in MS-100. The temperature of 22° to 25° C. was decided upon to reduce markedly cellular metabolism and reproduction.

Observations which were made prior to the performance of this experiment showed that L strain cells could be preserved in MS-100 at 22° to 25° C. for periods of 10 to 12 days. The appearance of cells from a culture which was maintained in this environment for 11 days is shown in Figure 3. It is evident by comparison of these cells with normal cells (Fig. 1) that cells under cultivation in MS-100 at 22° to

25° C. contain less cytoplasm than L strain cells in a serum embryonic extract mixture at 37° C. Successful maintenance of cells has not been obtained as consistently at 22° to 25° C. with either UF-33 and H-67, or MS-100. However, at 37° C., L strain cells were maintained as well in MS-100 as in ox serum ultrafiltrate medium. This finding suggested that it was the incubation temperature range of 22° to 25° C. that resulted in the less consistent maintenance of L strain cells in



Text-fig. 4. Results from experiment 4. The effect of MS-100 medium and lowered temperature (22° to 25° C.) on the cultivation of pseudorabies virus in cultures of L strain cells.

MS-100 and not the composition of the medium. However that may be, cells from many cultures maintained in MS-100 at 22° to 25° C. were transferred successfully following re-exposure to HoS-40, EE-20, H-40 at 37° C. for 2 or 3 days.

Experiment 4. The procedure employed for experiment 4 was similar to that described for experiment 3 except that MS-100 at 22° to 25° C., replaced UF-33, H-67, at 37° C.

The results from experiments 4A and 4B are shown in Text-figure 4. In contrast to those obtained for serum-embryonic extract medium or for ox serum ultrafiltrate at 37° C. (experiments 1 and 3), the results of this experiment do not clearly demonstrate viral multiplication. The curve for experiment 4A (Text-fig. 4) shows a gradual decline in the amount of virus present in the liquid phase of the culture. This result may signify merely that virus survived. The relatively constant level of virus in the liquid from the culture represented by curve B suggests

that an equilibrium between the rates of viral deterioration and viral multiplication may have existed during the first 6 days of cultivation. Therefore, some viral multiplication may have occurred. Microscopic observations of the cells in these cultures revealed a gradual reduction in the population of cells. Since cellular proliferation was minimal at this temperature, the cellular population was not replenished. It seems probable that the inadequate evidence for significant viral multiplication in these experiments resulted from this diminution of the number of viral host cells in the cultures.

Demonstration of Inclusion Bodies in the Nuclei of L Strain Cells Infected with Pseudorabies Virus

The infection of cells with pseudorabies virus commonly results in the presence of type A inclusion bodies within the nuclei of cells. Experiment 5 was performed to determine whether inclusion bodies occurred in L strain cells which were infected with pseudorabies virus.

Experiment 5. To each of six Porter flasks, which contained coverglasses, 11 by 22 mm., and abundant L strain cells, was added pseudorabies virus, 0.1 ml. and fresh HoS-40, EE-20, H-40, 0.9 ml. On the third, fifth, and seventh days of incubation at 36 to 37° C., the coverglasses from two flasks were removed. The cells were fixed with Bouin's solution and stained with Harris' hematoxylin and eosin.

Type A intranuclear inclusion bodies were found in L strain cells which were stained after 3 and 5 days of viral cultivation (Fig. 4). Scattered isolated cells and cells in small groups contained inclusion bodies. However, most cells did not contain inclusions.

OBSERVATIONS WITH HERPES SIMPLEX VIRUS

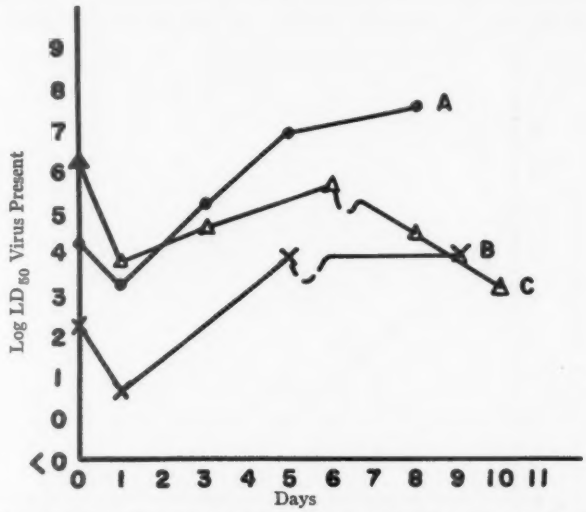
To extend the findings which showed that L strain cells support the growth of pseudorabies virus, the virus of herpes simplex was employed for experiments 6 through 10. Essentially similar procedures were utilized for these experiments as for experiments 1 to 5 with pseudorabies virus.

Cells Cultivated in Horse Serum-Embryonic Extract Medium at 37° C.

Physiologically active L strain cells cultivated in horse serum-embryonic extract medium were employed for the propagation of herpes simplex virus in three experiments.

Experiment 6. The procedure for experiments 6B and 6C was identical to that used for experiment 1. For experiment 6A, HoS-40, EE-20, H-40 was utilized instead of HoS-40, EE-10, H-50, and on the third, fifth, and eighth days after viral inoculation coverslips were removed from two flasks for examination of the cells after staining with hematoxylin and eosin. From each remaining flask, fluid, 0.25 ml., was removed, pooled with the fluids from other flasks, and analyzed for viral content. No fresh medium was added to the flasks in experiment 6A during the 8 days of viral cultivation.

Text-figure 5 presents the results from experiments 6A, 6B, and 6C. It can be seen that for experiments 6A and 6B, herpes simplex virus grew in physiologically active L strain cells. It is evident that these cultures yielded more virus than was inoculated. The shape of curve C (experiment 6C) suggests that virus multiplied, since it is similar in shape to those from experiments 6A and 6B. However, the amount of virus present in the liquid phase of the culture for experiment 6C did not exceed the quantity of virus present immediately following inoculation.



Text-fig. 5. Results from experiment 6. The growth of herpes simplex virus when measured in the liquid phase of L strain cultures which employed HoS-40, EE-10 or EE-20, H-50 or H-40 medium at 37° C.

Survival of Herpes Simplex Virus in Cell-Free Medium at 37° C.

As a control for the results from experiment 6, the survival of herpes simplex virus in the absence of cells was determined.

Experiment 7. The procedure for experiment 7 was that of experiment 2 except that fresh HoS-40, EE-10, H-50 was employed rather than media which had been removed from cultures of normal L strain cells and rendered free from cells by centrifugation.

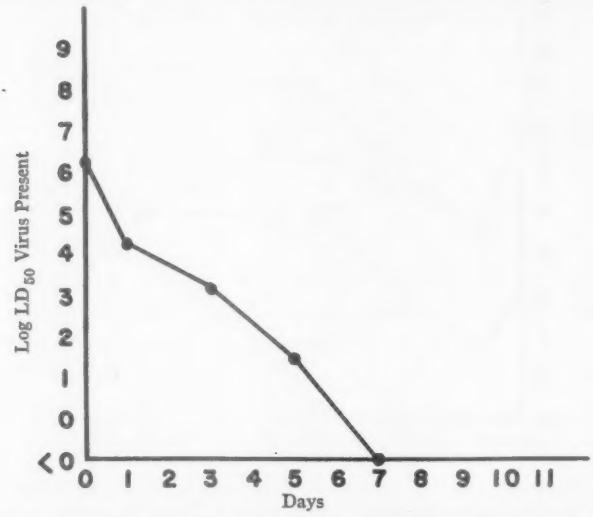
Text-figure 6 shows that following the inoculation of a large quantity of herpes simplex virus, $10^{6.3}$ LD_{50}, into cell-free HoS-40, EE-10, H-50 medium at 37° C., virus progressively decreased in quantity and disappeared by the seventh day. The presence of virus past the seventh day in experiment 6 must have resulted, therefore, from the presence of cells in the cultures and presumably from the multiplication of virus within the cells.

Cells Cultivated in Ox Serum Ultrafiltrate Medium at 37° C.

L strain cells which were maintained at 37° C. in UF-33, H-67 medium were employed.

Experiment 8. The procedure for experiment 8 was similar to that used for experiment 3.

The results from experiments 8A and 8B are shown in Text-figure 7. Although the quantities of virus which were present in the liquid phases of the cultures never exceeded the amounts of virus present immediately following inoculation, the curves suggest that virus multiplied. Moreover, in contrast with the observation that virus deteri-



Text-fig. 6. Results from experiment 7. The survival of herpes simplex virus in cell-free medium.

orated in the absence of cells (experiment 7), the results of these experiments show a definite increase in virus after the initial decrement which occurred in the first 24 hours of cultivation. This rise in quantity of virus is attributable, by comparison with the curve for experiment 7, to the presence of cells and presumably to viral multiplication within them. However, it is evident that the extent of viral multiplication in these experiments, which employed ox serum ultrafiltrate medium, was significantly less than that obtained by the use of horse serum-embryonic extract medium (experiment 6).

Cells Cultivated in Maintenance Solution at 22° to 25° C.

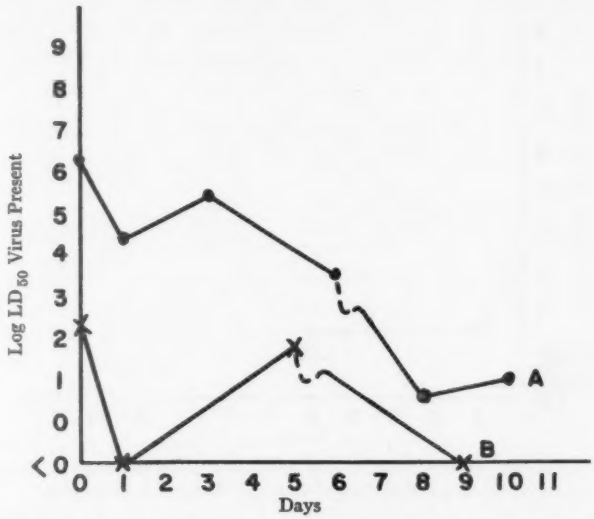
The propagation of herpes simplex virus was studied in cultures of L strain cells maintained in MS-100 at 22° to 25° C.

Experiment 9. The procedure for experiment 9 was similar to that employed for experiment 4.

The results from experiment 9 (Text-fig. 8) show that either minimal or no multiplication of herpes simplex virus occurred in L strain cells which were maintained in MS-100 at 22° to 25° C.

Demonstration of Inclusion Bodies in the Nuclei of L Strain Cells Infected with Herpes Simplex Virus

Experiment 10 was performed to learn whether type A intranuclear inclusion bodies occurred in L strain cells infected with herpes simplex virus.



Text-fig. 7. Results of experiment 8. The growth of herpes simplex virus when measured in the liquid phase of L strain cultures which employed UF-33, H-67 medium at 37° C.

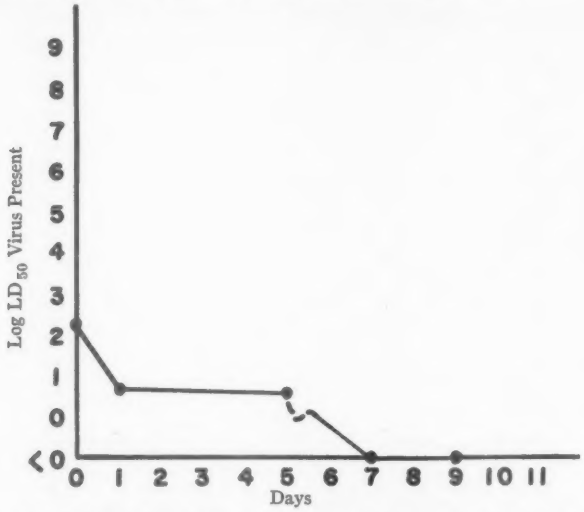
Experiment 10. The techniques as described for experiment 5 were utilized for experiment 10. Cells were stained on the third, fifth, and eighth days of viral cultivation. The growth curve of virus in this experiment is the curve labeled A in Text-figure 5.

Intranuclear inclusion bodies were found in L strain cells (Figs. 5 and 6). Occasionally, large multinucleated cells contained intranuclear inclusion bodies (Fig. 5). Most nuclei contained no inclusion bodies. However, the chromatin material in the nuclei of many cells in infected cultures (Fig. 7) appeared to be less dense than in the nuclei of normal cells (Fig. 1).

Serial Passage of Herpes Simplex Virus in Cultures of L Strain Cells

Additional evidence for the multiplication of herpes simplex virus in cultures of L strain cells was secured by ten successive serial passages

of the virus at weekly intervals. The experimental procedures were similar to those employed for experiment 1, with the exceptions that the liquid medium employed during the period of viral cultivation was HoS-40, H-60, instead of HoS-40, EE-10, H-50, and that no liquid medium was either added or withdrawn from the cultures during a single passage. At the end of each 7-day period of viral cultivation, liquid medium, pooled from two flasks, was inoculated into fresh cultures of L strain cells, and was titrated for viral content in mice.



Text-fig. 8. Results of experiment 9. The effect of MS-100 medium and lowered temperature on the cultivation of herpes simplex virus in cultures of L strain cells.

The data from this passage series are shown in Table II. It can be seen that herpes simplex virus was propagated successfully in cultures of L strain cells over a period of 70 days. The inoculum of herpes simplex virus that was utilized to initiate this series was liquid medium harvested from infected L strain cultures. It is apparent that the cumulative dilution factors that resulted from the serial passage of virus had surpassed the LD_{50} end point of the viral inoculum as early as the fourth viral passage. Moreover, the LD_{50} values for successive passages, when compared with the tenfold dilutions of virus that occurred with each passage, show unequivocally that virus multiplied.

Table III in summary of experiments 1, 3, 4, 6, 8, and 9 shows the yields that resulted when the viruses of pseudorabies and herpes simplex were propagated in cultures of L strain cells. It can be seen that from all but one of the cellular cultures with horse serum-embryonic extract medium more virus was recovered than was inoculated. With

UF-33, H-67 medium at 37° C., positive yields of pseudorabies virus, but not of herpes simplex virus, were obtained. No positive yields of either virus resulted from the cultures which employed MS-100 at 22° to 25° C.

Discussion

The results of these studies show that a pure strain of cells (L strain, Earle), derived from mouse subcutaneous tissue, supports the multiplication of pseudorabies and herpes simplex viruses. Two findings were accepted as proof for this statement: Each virus was recovered from cultures in quantities larger than were inoculated; type A intranuclear inclusion bodies were found in cells from cultures which were inoculated with either virus. Moreover, it was found that these viruses multiplied best in physiologically active cells maintained at 37° C. in

Table II

Propagation in Vitro of Herpes Simplex Virus in Cultures of Altered Mouse Fibroblasts (L Strain, Earle)

Number of virus passage	Total days in culture	Cumulative log of dilution of original viral inoculum	Result of mouse titration as negative log of LD_{50}/0.05 ml. of cultural liquid
Inoculum			3.9
1	7	1.0	4.6
2	14	2.0	4.2
3	21	3.0	3.4
4	28	4.0	4.5
5	35	5.0	3.4
6	42	6.0	3.5
7	49	7.0	1.7
8	56	8.0	1.5
13*	91	13.0	6.0
18	126	18.0	4.7

* The identity of the virus was established as herpes virus by the neutralization technique.

a medium which contained horse serum and chicken embryonic extract. Viral multiplication also occurred in cultures of cells which were maintained in a less active metabolic and proliferative state by the use of ox serum ultrafiltrate at 37° C. The yields of virus from these cultures, however, were significantly less than those obtained with horse serum embryonic extract medium. When the rates of cellular metabolism and proliferation were reduced markedly by cultivating the cells at 22° to 25° C. in a maintenance solution, multiplication of these viruses was either minimal or did not occur.

The occurrence of type A intranuclear inclusion bodies in L strain cells is taken as evidence that the cells were parasitized by the viruses

of pseudorabies and herpes simplex. The scarcity of cells which contained inclusion bodies, even though virus was being actively produced, is of unknown significance. However, the observation that large yields of virus occurred when relatively few cells showed inclusion bodies, and the finding that the nuclei of many cells in infected cultures were abnormal, suggest that viral production was not limited to cells which contained inclusion bodies.

The advantages of the utilization of a pure strain of cells for the cultivation of viruses *in vitro* in preference to the mixed cellular populations of tissue fragments commonly employed[13] are many. Several limitations to the successful use of mixed cellular cultures for the study of mammalian cell-virus relationships are worthy of discussion.

In tissue cultures there co-exist viral host cells and cells which do not participate in viral reproduction. Hence, the accurate identification and enumeration of viral host cells, and the precise determination

TABLE III

Summary of Viral Yields from L Strain Cultures (Experiments 1, 3, 4, 6, 8, and 9). The Effects of Temperature and the Composition of the Medium on the Growth of Pseudorabies and Herpes Simplex Viruses in Cultures of L Strain Cells

Virus	Experiment	Cultural medium and temperature of incubation		Quantity of virus		
				Recovered	Inoculated	Produced
		°C.		LD_{10}	LD_{10}	LD_{10}
Pseudorabies	1A	37	HoS-40*, EE-10, H-50	326,011,600	12,600	325,999,000
	1B		HoS-40*, EE-10, H-50	22,350,500	1,260	22,349,240
	3A	37	UF-33, H-67†	35,180	12,600	22,580
	3B		UF-33, H-67†.	16,680	1,260	15,420.
	4A	22–25	MS-100‡	1,335	12,600	0
	4B		MS-100‡	190	1,260	0
Herpes simplex	6A	37	HoS-40, EE-20, H-40	45,000,200	20,000	44,980,200
	6B		HoS-40, EE-20, H-40	15,151	200	14,951
	6C		HoS-40, EE-10, H-50	549,100	2,000,000	0
	8A	37	UF-33, H-67	109,464	2,000,000	0
	8B		UF-33, H-67	>60	200	0
	9A	22–25	MS-100	<10	200	0

* HoS-40, EE-10, H-50 denotes a mixture of horse serum, 40 per cent, chicken embryonic extract, 10 per cent, and Hanks' salt solution, 50 per cent.

† UF-33, H-67 signifies a mixture of ox serum ultrafiltrate, 1 part, and Hanks' solution, 2 parts.

‡ MS-100 indicates undiluted maintenance solution.

of the degree of viral reproduction in each existent cellular type are made difficult or impossible to achieve. Moreover, for studies of viral metabolism in mammalian tissue cultures, the physiologic activities of

cells which are not viral host cells are difficult to distinguish from the metabolic activities of viral host cells.

The physical substrate, such as clotted plasma, cellophane,[7] or cellulose sponge,[14] which mammalian cells usually require for cellular proliferation, may limit the use of tissue cultures for studies of virus-host cell relationships. For example, if it is necessary to obtain a suspension of cells, the substrate must be removed, and the use of proteolytic enzymatic digestion to release cells from clotted plasmas[15-17] may be detrimental to the cells or to the virus. Moreover, plasma contributes nutritive materials to cells and thus limits the accuracy of metabolic studies when measurements of metabolites are limited to the liquid phase of cultures. The plasma coagulum may also affect the degree of exposure of cells to substances, such as viruses, in the liquid medium, for cells often grow at multiple levels in the substrate.

Cells in tissue cultures are not of defined heritage. With the exception of one cell strain (L strain, Earle[4]), no cultures of mammalian cells which originated from a single cell have been reported. Thus, mammalian viruses in tissue cultures, unlike bacterial viruses, have not been studied in homogeneous populations of cells.

For quantitative studies of viral reproduction, precise enumeration of viral host cells and the preparation of large numbers of replicate cultures are often essential. Yet, when fragments of tissue are employed, the enumeration of cells and the reproduction of identical cultures are, at best, inaccurate procedures.

These limitations to the study of virus-host cell relationships which are inherent in the use of ordinary tissue cultures can be surmounted by the employment of pure strains of cells such as the L strain. These cells were derived from a single cell, grow readily in a single-cell layer on glass surfaces in the absence of plasma, can be enumerated accurately by counting cellular nuclei, and can be prepared as replicate cultures. These unique characteristics of L strain cells make them particularly useful for viral studies. The propagation of either pseudorabies virus or herpes simplex virus in cultures of these cells provides a mammalian virus-cell system which is closely analogous to the bacteriophage-bacterial cell system. It should be possible, therefore, to study quantitatively the adsorption, penetration, metabolism, and reproduction of these viruses in cultures of L strain cells by employing methods similar to the methods which have been utilized for studying bacteriophage.

Another aspect of this investigation was the study of the effects upon viral reproduction of the metabolic and proliferative activities of the

cells. It is probable that these relationships between viruses and cells vary among different cell-virus systems. For example, at room temperature (23° to 30° C.) the viruses of lymphogranuloma venereum, St. Louis encephalitis, and Western equine encephalomyelitis propagate in embryonic chicken tissue cultures[18-20] and epidemic keratoconjunctivitis virus multiplies in cultures of embryonic mouse brain.[21] Cellular metabolism and proliferation are minimal at those temperatures. In contrast to the growth of viruses in physiologically inactive cells are the results of studies which showed that Western equine encephalomyelitis virus multiplies maximally during the period of active metabolism of chicken embryonic tissue, when the rate of metabolism was measured by oxygen consumption of the tissues.[22] Thus, generalizations from results obtained with one virus cultured in one type of tissue or cell are not justified.

The results of the present studies show that significant differences occurred in the production of pseudorabies and herpes simplex viruses by L strain cells which were maintained at three levels of physiologic activity. Explanations for these differences in yields of pseudorabies and herpes simplex viruses are not, however, entirely evident. It was observed that the addition of either virus to L strain cells resulted in a reduction of the cellular population. In horse serum-embryonic extract medium, in which cells were proliferating, the net decrement in the number of living cells was minimal. On the other hand, in cultures which employed MS-100 at 22° to 25° C., in which the rate of cellular proliferation was less than the rate of cellular death, the population of cells steadily diminished. It is possible that the lack of significant viral multiplication in the latter cultures may have been related, in part, to this progressive decrease in the population of cells. Destruction of cells was related to the presence of virus, for it was shown that a suspension of normal mouse brain tissue did not destroy cells which were being maintained either at 37° C. in ox serum ultrafiltrate, or at 22° to 25° C. in maintenance solution. It seems, therefore, that both the rates of cellular proliferation and of cellular destruction are important factors which determine the yields of these viruses from cultures of L strain cells.

Summary

A pure strain of altered mouse fibroblastic cells (L strain, Earle), derived from a single cell, was found to support the multiplication of pseudorabies and herpes simplex viruses *in vitro*. Type A intranuclear inclusion bodies occurred in L strain cells infected with either virus. The production of virus in cultures of these cells was greatest when

the cells were maintained in a physiologically active state by the use of a horse serum-embryonic extract medium at 37° C. Less virus was obtained from cultures that contained cells kept at a lower level of metabolic activity by the use of diluted ox serum ultrafiltrate at 37° C. Viral production was minimal, or not detectable, in cultures in which the cells were in a state of apparent metabolic and reproductive inactivity, consequent to the utilization of a maintenance solution at 22° to 25° C.

The photographs were made by Mr. Henry Morris. The technical assistance of Miss Elizabeth Garbutt and Mrs. Joanne Herman is acknowledged.

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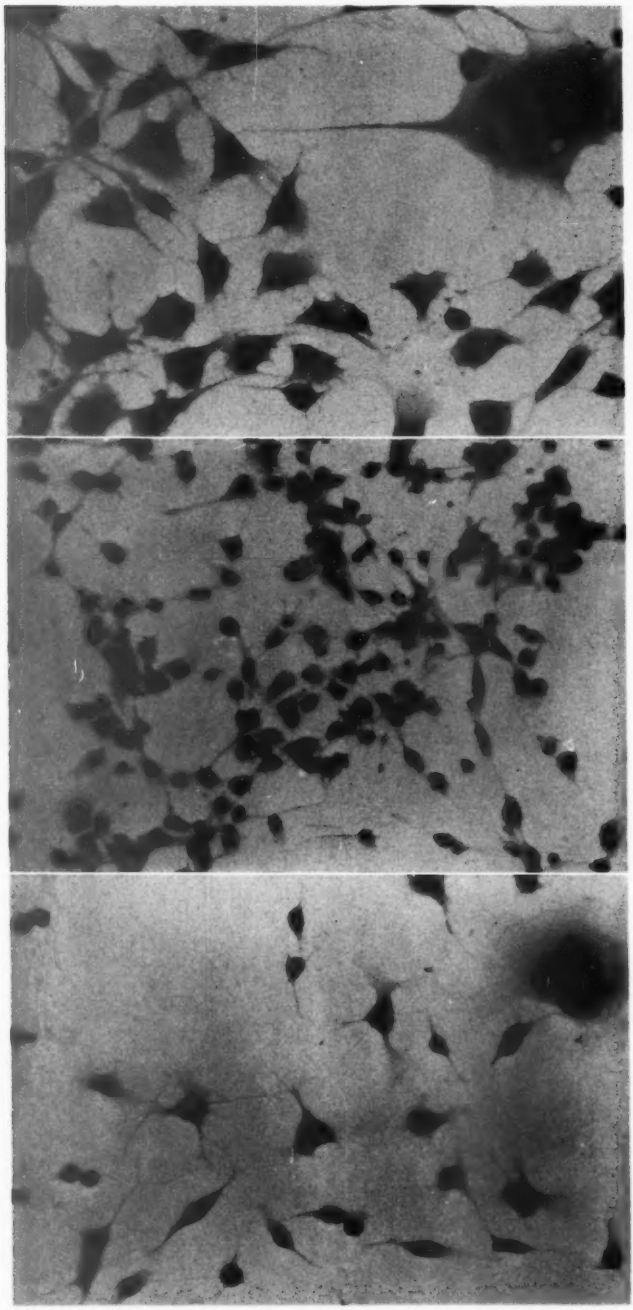
LEGENDS FOR FIGURES

The appearance of L strain cells cultivated in the absence of virus. Harris's hematoxylin and eosin stain.

FIG. 1. Normal L strain cells cultured for 7 days in HoS-40, EE-20, H-40 medium at 37° C. × 375.

FIG. 2. L strain cells maintained in UF-33, H-67 medium at 37° C. for 11 days following a preliminary growth period of 7 days' duration in HoS-40, EE-20, H-40 medium at 37° C. × 375.

FIG. 3. L strain cells maintained in MS-100 medium at 22° to 25° C. for 11 days following a preliminary growth period of 7 days' duration in HoS-40, EE-20, H-40 medium at 37° C. × 375.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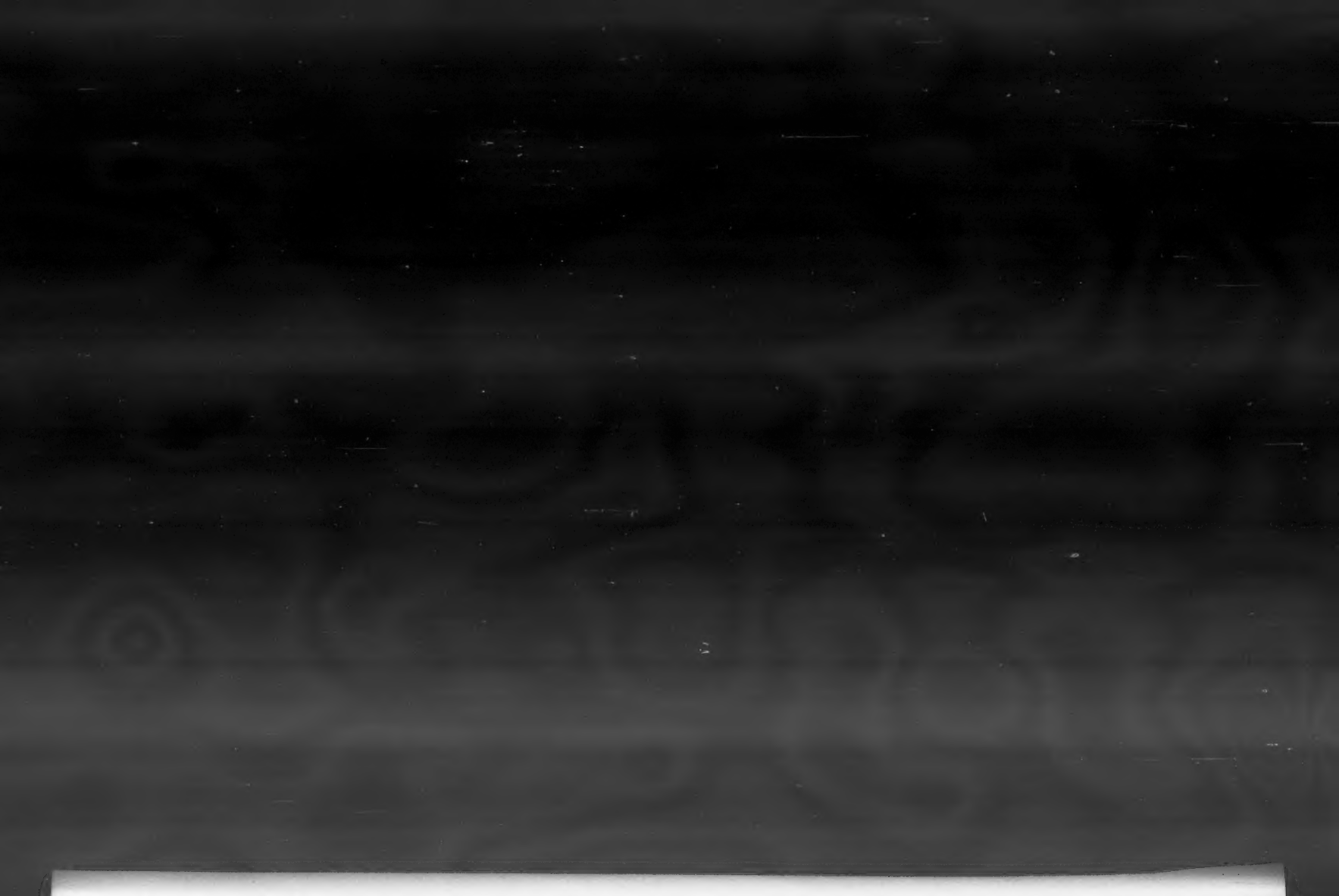
The appearance of L strain cells cultivated in the presence of the viruses of pseudorabies or herpes simplex. Harris's hematoxylin and eosin stains.

FIG. 4. Cells which were cultured for 5 days in HoS-40, EE-20, H-40 medium with pseudorabies virus show type A intranuclear inclusion bodies, indicated by the arrows. × 800.

FIG. 5. Numerous inclusion bodies in nuclei of a multinucleated cell are illustrated. Nearby smaller nuclei also contain inclusion bodies. × 800.

FIG. 6. Cells which were cultured in HoS-40, EE-20, H-40 with herpes simplex virus for 3 days contain type A inclusion bodies in adjacent nuclei. × 800.

FIG. 7. Cells which were cultured in HoS-40, EE-20, H-40 for 3 days after inoculation of herpes simplex virus show nuclear chromatin which is less compact than the chromatin in the nuclei of normal cells (Fig. 1). × 375.



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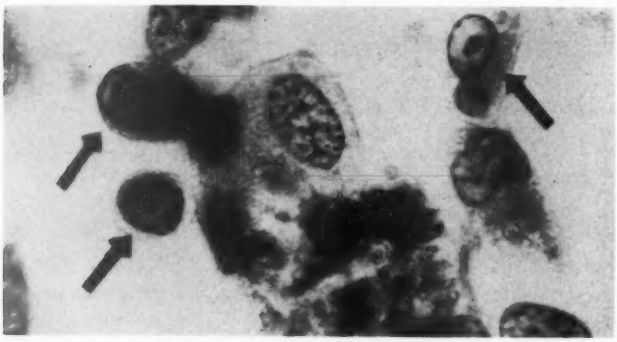

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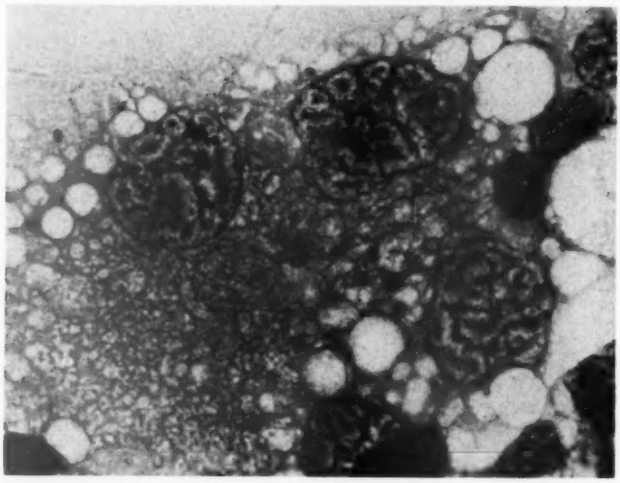

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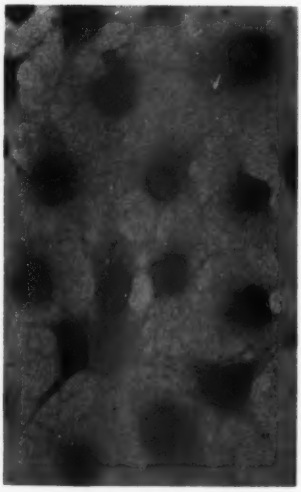

CULTIVATION OF EQUINE ABORTION VIRUS IN FETAL HORSE TISSUE IN VITRO *

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Equine virus abortion was first described as a clinical entity by Dimock and Edwards.[1] Subsequent reports[2-8] established its viral etiology and adequately characterized the malady as an epizootic contagious disease of Equidae. It is characterized by abortion, usually in the late stage of pregnancy, without signs of infection in the mare before or after parturition. The striking pathologic lesions noted in the aborted fetus are small foci of necrosis in the liver, hemorrhages in the abdominal and thoracic organs, edema and congestion of the lungs, and pleural effusion.

The host range of the virus appears to be narrowly limited. Histopathologic evidence of infection in newborn Syrian hamsters and in human placental tissue has been adduced by Goodpasture and Anderson.[9,10] Other investigators[11] have not succeeded in demonstrating infection in day-old hamsters. Dimock, Edwards, and Bruner[6] were not successful in inducing infection in embryos of the mouse, rabbit, guinea-pig, or chick. Contrary to these reports, we have noted intranuclear inclusions in tissue cultures of the spleens of the day-old hamster and day-old dog,[12] but these effects could not be reproduced in serial passage.

Since a supply of horse tissue was accessible, it seemed of interest to attempt to cultivate the causative agent in tissue culture. The results of our studies are presented here. The evidence rests on the demonstration of inclusions, the results of complement fixation, and animal inoculation.

TISSUE CULTURE, MATERIALS AND METHODS

General. Fetal horse tissue was obtained from 9 mares, ranging from 6 to 11 months' gestation. Fetal tissues were removed with scrupulous attention to aseptic techniques. Portions of lung, liver, spleen, chorio-allantoic membrane, amnio-allantoic membrane, and testis were removed from the first two fetuses for tissue culture. Also representative portions were fixed in Zenker-acetic acid for control studies. Preliminary histologic examination of cultured tissues indi-

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cated that only spleen and lung were satisfactory as far as inclusions were concerned; therefore, in subsequent experiments only these tissues were employed. Tissues were cultured in flasks by the Maitland[13] procedure and by the coverglass method. Chick embryo extract and chicken plasma were prepared by conventional methods. The nutrient phase for the flask cultures consisted of equal parts of human ascitic fluid and Hanks' balanced salt solution. That for the slide preparations was composed of 50 per cent human ascitic fluid, 47 per cent balanced salt solution, and 3 per cent chick embryo extract. Five hundred units of penicillin were added per ml. of nutrient fluid. All materials were subject to strict bacteriologic control. The virus-containing inoculum for the tissue culture preparations was the same infected foal tissue used throughout the development of the complement fixation test for equine virus abortion which has previously been described.[14] Briefly, it consisted of a high-speed centrifugate of infected foal lung diluted 1:2 with physiologic saline solution, sealed in ampoules and stored at —40° C.

The various tissues used in both the flask and coverglass methods were cut into cubes of approximately 2 mm. and rinsed three times with balanced salt solution.

Flask Method. Tissue from the first two fetuses consisted of spleen, lung, liver, testis, amnio-allantoic and chorio-allantoic membrane. Subsequent experiments utilized only spleen and lung for reasons already noted. Duplicate sets of spleen were prepared, one set, together with lung, being inoculated with the standard stored virus, the other set with serial passage material. For each tissue nine flasks were arranged, three serving as controls. Routinely ten to fifteen pieces of tissue with a minimum amount of fluid (averaging 0.3 ml.) were transferred with a 5 ml. pipette to a rubber-stoppered 50 ml. Erlenmeyer flask containing 3 ml. of nutrient fluid, pH 7.6. In addition to the routine procedures, a large number of flasks, containing 75 mg. each of spleen, were prepared from the eighth fetus. Preliminary experiments indicated that with ten to fifteen pieces of tissue the pH could be maintained without significant change. However, with a larger amount of tissue, a drop in pH to about 6.8 on approximately the fourth day of incubation was an indication for complete exchange of nutrient material. At the end of 24 hours' incubation at 37° C. the test flasks were inoculated with virus-containing material, each new set of lung and one of spleen receiving 0.05 ml. of a 50 per cent suspension of standard stored virus. The other set of flasks containing spleen were inoculated with passage virus, which was prepared by pooling the cultured tissue, freezing, and grinding to a smooth paste.

The resulting material was resuspended in pooled nutrient fluid, adjusting the amount so that the dilution of each serial passage was 1:66. The homogeneous suspension and aliquots of pooled nutrient fluid were sealed separately in ampoules and stored at —40° C. All cultures were incubated routinely from 6 to 8 days. Representative tissue fragments were removed from each culture series at intervals from 3 to 7 days after inoculation and fixed in Zenker-acetic acid mixture.

Coverglass Method. At the initiation of these studies the same tissues used in the flask experiments were also explanted in plasmatic medium. Preliminary observation indicated that fetal spleen and lung, and the amnio-allantoic membrane were the most satisfactory from the standpoint of growth; consequently, observations were confined to these tissues. Cultures from the first five fetuses were made by the method outlined. Three or four pieces of tissue were embedded in plasma clot (equal parts of chicken plasma and chick embryo extract) on a no. 1 coverglass, 12 by 50 mm. The preparation was then placed in a 16 by 150 mm. pyrex test tube, and after standing for 1 hour, 2 ml. of nutrient fluid were added to each tube and the cotton plugs replaced by sterile no. 1 rubber stoppers. The preparations were slanted in a basket at an angle, barely allowing the liquid phase to cover the surface of the coverglass. After 24 hours of incubation, virus-containing material in 0.05 ml. amounts was added to the cultures. In addition, duplicate sets of lung and spleen were prepared in which the same amount of virus in 0.5 cc. of balanced salt solution was mixed thoroughly with the tissue and allowed to stand at 4° C. for 2 hours before explanting. All tubes were observed daily for bacterial contamination and pH change. Cultures with suitable controls were fixed in Zenker-acetic acid and stained with hematoxylin-eosin-azure after 4, 6, and 8 days of incubation.

Experimental Animals, Inoculation, and Bleeding. In order to test the specificity of the virus, apparently capable of inducing inclusions in tissue culture, 10 mares at approximately 7 months' gestation were selected for inoculation. The experiment was designed for two purposes: to measure the complement-fixing antibody response, and to determine if the virus had been modified by tissue culture passage, or was still capable of causing abortion and lesions characteristic of the disease in question. Control blood (noted as 0 days in Table I) was drawn immediately before intravenous injection of 35 ml. of eighth passage tissue culture material, including exchange nutrient fluid. Each animal received approximately 0.8 gm. of cultured fetal spleen; the final dilution of virus being $2(66)^8$ or $10^{14.8}$ in terms of the orig-

inal virus-containing inoculum. Blood was collected 2, 4, 6, 8, 10, 12, 14, 20, and 30 days after inoculation. The clarified serum samples were handled aseptically and stored in cork-stoppered tubes at —18° C.

Complement-Fixation Methods. The original complement-fixation test for virus of equine abortion was developed by Randall, McVickar, and Doll,[14] employing the 50 per cent hemolysis end-point technique according to the spectrophotometric method developed by Mayer *et al*,[15] and Kent *et al.*[16] This method was used to determine the antibody response in pregnant mares following injection of tissue culture materials and to determine the complement-fixing activity of tissue culture virus. The various sera and antigens used in the development of the original procedure had been sealed in ampoules and stored in small amounts at —40° C. Representative samples were tested every 1 to 3 months over a period of 2 years and appeared to have undergone no significant change in titer and were used as reference material in the current investigation. Since the method has already been published in detail, only the bare essentials will be noted here. The optimal dilution of standard antigen and serum had been determined to be 1:100 and 1:20 respectively. In determining the antibody titer of mares' sera, mixtures of equal parts (1 ml. each) of antigen (1:100), two-fold dilutions of mare's serum and complement (containing $3H_{50}$ units) were allowed to stand at 4° C. for 18 hours. At the end of this primary period, 1 ml. of optimally sensitized sheep erythrocytes was added to the system, followed by a 45 minute period of incubation at 37° C. After centrifugation of the tubes, the test was read immediately, and a positive reading was considered to be one in which 80 per cent hemolysis or less occurred, and which was not anti-complementary. The end-point is expressed in terms of initial dilution of the test material before the addition of the various reagents. Adequate controls, including normal fetal horse spleen and lung and normal nutrient fluid, were included in each test.

The tissue culture antigen (except for fifth passage material in which physiologic saline solution was substituted for nutrient fluid) was prepared by freezing and grinding the pooled splenic tissue and resuspending in pooled nutrient fluid. The resulting mixture and separate aliquots of nutrient fluid used to nourish the tissues were centrifuged at 13,500 r.p.m. for 30 minutes at 4° C. The resulting clear supernatant was sealed in ampoules and stored at —40° C., until ready for use. In determining the complement-fixing activity of tissue culture material, serial two-fold dilutions were titrated against a fixed quan-

tity of positive serum (1:20). The remainder of the procedure was identical with that carried out in the serum titrations.

Experimental

Flask Method. All tissues were stained with hematoxylin and eosin. The intranuclear inclusions were not recognized in amnio-allantoic or chorio-allantoic membrane or testis. The placental membranes were well preserved, but the tubules of the testis had largely degenerated. The liver sections showed complete degeneration of the parenchymal cells; however, the bile ducts were well preserved and in many instances had proliferated. A very rare inclusion was present in a well preserved Kupffer cell.

Sections of lung and spleen were most satisfactory both from the standpoint of development of inclusions and preservation of tissue (Figs. 1 to 4). Preparations were fixed and stained 3 to 7 days after inoculation (or 4 to 8 days of incubation). These revealed numerous intranuclear acidophilic to neutrophilic inclusions, usually of the type surrounded by a clear halo with margination of chromatin. However, occasional inclusions were seen both in spleen and lung (more commonly in the latter) in which the inclusion appeared to fill the nucleus. In the spleen, inclusions were most evident in cells of reticulo-endothelial type without respect to any particular location. Certain of the splenic tissues showed hyperplasia, usually focal, but occasionally diffuse, and were made up of cells not unlike endothelial cells in appearance, or more closely resembling reticulated fibroblasts. Inclusions were found in these cells as well, and occasionally in small, dark, round cells resembling lymphocytes. With serial passage through splenic tissue, inclusions became quite numerous and, in some instances, practically every cell appearing viable contained these bodies. As a result of serial passage, the original virus inoculum had been diluted $10^{16.8}$ times. The lung sections were generally in an excellent state of preservation, on the whole better than the spleen. Inclusions were found in large, plump, oval to spindle cells between the alveolus-like structures and in their lining cells, and in the epithelial cells of the bronchioles. Unfortunately, these findings were not constant, the lung of one fetus showing numerous inclusions, another being almost devoid of them. Necrosis, usually focal in nature, was evident in sections of both lung and spleen without regard to the presence of inclusions. Moreover, no significant differences could be distinguished from the control tissues in respect to necrosis. No inclusions were ever recognized in the numerous controls.

Coverglass Method. The numerous explants of spleen, lung, and amnio-allantoic membrane inoculated with virus in the liquid phase after 24 hours of incubation showed excellent growth which, in the case of spleen and lung, appeared predominantly fibroblastic. For the membrane, the outgrowth was composed of a thin sheet of regular polygonal cells, resembling epithelium. Control tissues showed no significant differences in any of the preparations.

Other efforts dealt with the growth of explants placed in intimate contact with the virus before being incorporated in the clot. In this instance the outgrowth might be delayed 1 to 2 days and was often

TABLE I

Complement Fixation Titer of Sera from Pregnant Mares Inoculated Intravenously with Eighth Passage Tissue Culture Virus

Mare no.	Control serum	Specified days post-inoculation				
		4	8	12	20	30
238	0	0	256	128	64	64
240	0	16	128	256	128	256
241	0	0	256	64	128	64
242	0	64	512	512	256	128
243	0	64	256	256	64	64
246*	16	64	512	256	128	64
247	16	32	256	128	128	128
248	0	16	256	256	256	128
249*	16	32	512	512	256	128
250	0	0	256	512	256	128
Average titer	4.8	28.8	320.0	288.0	166.4	115.2

* 246 and 249 aborted after 69 and 14 days respectively, the fetuses showing typical lesions of viral infection.

scanty. Occasionally the growth was inhibited entirely. Some, however, showed no differences from the excellent growth manifested by the controls. Careful examination of the more than 100 explants cultured by the methods outlined revealed not a single instance of a recognizable inclusion. The preparations viewed through plasma, though adequate, left something to be desired from the technical standpoint.

Experimental Disease in Animals Receiving Tissue Culture Virus. Following intravenous injection of eighth passage material, two of ten pregnant mares aborted on the 14th and 69th day respectively (Table I). It should be noted at this point that in terms of the original virus-containing tissue, the tissue culture inoculum represented a dilution of $10^{14.8}$. The lesions in the lung, liver, and spleen of the aborted

fetuses were typical (Figs. 5 and 6). The focal necrotic lesions in the liver were not numerous, but were characteristic. Inclusion bodies in association with these areas were infrequent. The splenic follicles exhibited fairly uniform degeneration and necrosis. Inclusion bodies were relatively frequent in the spleen and the lung.

These results should be compared with those of Doll,[17] who inoculated 22 pregnant mares with a variety of field strains of equine abortion virus. According to this investigator, only 3 abortions resulted.

Antibody Response to Inoculation of Tissue Culture Virus. Examination of Table I will show a significant rise in levels of complement-fixing antibody following inoculation of eighth passage virus. The

TABLE II

Complement Fixation of Tissue Culture Virus and Comparison with Original Virus-Containing Tissue

Antigen (passage no.)	Dilution relative to original virus-containing tissue	Dilution of virus as antigen in complement-fixation test						
		1/2	1/4	1/8	1/16	1/32	1/320	1/640
0*	2						+	−
1	2 (66)	A.C.	+	−	−	−		
3	$2\ (66)^3$	A.C.	A.C.	A.C.	−	−		
5	$2\ (66)^5$	A.C.	A.C.	+	+	−		
8	$2\ (66)^8 = 10^{14.8}$	A.C.	A.C.	+	−	−		

* Original virus-containing tissue.
A.C. = anti-complementary.
+ = 80% hemolysis or less.

figures noted in Table I represent the highest (initial) dilutions of serum showing 80 per cent hemolysis or less, and which were not anti-complementary. The titers for respective days post-inoculation were averaged and were noted to reach a maximum on the eighth day. It is interesting to observe that of 3 mares which initially had a low titer of antibody before inoculation, 2 aborted. For reason of brevity, the various controls are not indicated in Table I. Besides the usual standards, other controls consisted of standard stored positive and negative sera, normal fetal horse spleen, and lung antigen and normal nutrient fluid.

Comparison of Complement-Fixing Activity of Tissue Culture Virus and Original Virus

It is clear from examination of Table II that tissue culture virus serves as a complement-fixing antigen; albeit, in most instances, a rather weak one. The antigens were, unfortunately, rather anti-com-

plementary; but, with the exception of third passage material, this factor could be diluted out. For the sake of simplicity, the results with test nutrient fluid were not included in Table II. They were uniformly anti-complementary in low dilutions, and when this factor was removed by dilution no fixation of complement ensued, contrary to the experience with the ground antigen. It is clear that by use of tissue culture propagation of the virus has been demonstrated if one compares the different antigens. The original virus-containing material would not fix complement when diluted beyond 1:320 ($10^{2.5}$), in contrast to eighth passage tissue culture material. This antigen, which represents virus diluted 8 $(10)^{14.8}$ or $10^{15.4}$ fold with respect to the original virus was able to fix complement. This represents a $10^{12.9}$ fold increase in the amount of virus over the original inoculum.

Doll,[17] in an independent investigation, applied the conventional Kolmer technique to the problem of determining the complement-fixing properties of eighth passage, tissue-culture antigen. He was able to obtain serum from several yearling horses which had been in contact with mares who had aborted virus-infected foals. The yearlings developed complement-fixing antibodies, presumably as a result of contact exposure. He states that "against a weanling serum, titer previously determined to be 1:128, the tissue culture antigen in question resulted in complete fixation of 2 full units of complement at antigen dilutions of 1:4 and 1:8 with a fixed serum dilution of 1:10. With a 1:5 dilution of the same serum the tissue culture antigen fixed complement at a dilution of 1:16. These were simple titrations of antigen (two-fold) against a fixed quantity of positive serum. The tissue culture material was anti-complementary undiluted and at 1:2." These data are in close agreement with the results obtained by the 50 per cent spectrophotometric method. The results with serum from naturally acquired cases appear to offer further evidence that complement fixation with tissue culture antigen was not due to the horse protein which it may contain.

Discussion

In the studies here reported, it is clear that a rather restricted virus, the causative agent of equine abortion, has been cultured *in vitro* in fetal horse tissue. The typical intranuclear inclusions seen in the natural disease were reproduced regularly in spleen and lung inoculated in flasks. It is not clear why the necrosis seen in the various organs in the diseased fetus was not manifest in the tissue culture preparations.

Inclusions were present in fixed and stained tissues removed variously from 3 to 7 days after inoculation. The earliest appearance and duration of inclusion bodies in tissue culture are currently being investigated. It is challenging that histologic evidence of virus propagation, judging by inclusions, was manifest in suspended cell cultures in which proliferation may take place; but, if present, probably occurs at a much slower rate than in tissues explanted in plasmatic medium. In contrast, the latter preparations were never observed to contain inclusions, regardless of method of exposure of tissue to the virus or rate of cellular growth as manifest by the zone of outgrowth.

Mention has been made of the original complement fixation test for equine virus abortion. Stored aliquots of original virus were used as inoculum for the tissue culture preparations, and the antigens and antisera used in the original test were employed in the various serologic procedures. Thus a thread of continuity has been maintained among the various lines of investigation.

The tissue-culture virus, diluted $10^{14.8}$ times in terms of the original virus inoculum, was able to induce abortion with lesions and to provoke a high titer of complement-fixing antibody when injected into pregnant mares.

Perhaps the most conclusive evidence for the propagation of the virus revolves around the determination of the complement-fixing activity of tissue culture preparation. It has been conclusively shown by comparison with the original infected tissue that the virus has increased in titer $10^{12.9}$ fold over the original inoculum. The undesirable anti-complementary activity of the spleen could have been circumvented by the use of lung in serial passage, as this tissue has been shown by us to have little comparative anti-complementary effect; except that inclusions were not present as regularly in lung as in spleen.

The various serologic data, induction of inclusions in serial passage and reproduction of the natural disease appear to indicate conclusively that the specificity of the virus has been maintained. The hope that passage of the agent through tissue culture would induce modification, with the possibility of immunization without abortion, has not as yet been realized.

Summary

By means of tissue culture, the virus of equine abortion has been propagated through nine serial passages in fetal horse spleen by the Maitland method. By this procedure, the final dilution in terms of the original virus-containing tissue was $10^{16.8}$.

Intranuclear inclusions are produced regularly in fetal equine spleen and lung with excellent preservation of tissue. Inclusions have not been observed in tissue explanted in plasmatic medium.

Inoculation of eighth passage tissue-culture virus (original inoculum diluted $10^{14.8}$) into 10 pregnant mares induced 20 per cent abortion (consistent with field trials) and high levels of complement-fixing antibody.

The complement-fixing activity of tissue-culture virus was demonstrated, and showed that the virus in question had been propagated. In comparing original virus with eighth passage tissue-culture virus as antigen, it was shown that the latter was able to fix complement at a dilution $10^{15.4}$ times that of the former. This represents a $10^{12.9}$ fold increase in the amount of virus over the original inoculum.

These experiments indicate that the specificity of the virus of equine abortion has been maintained in tissue culture without apparent modification of its original properties. Further experiments are in progress attempting to adapt the *in vitro* propagated strain to tissues of other embryos.

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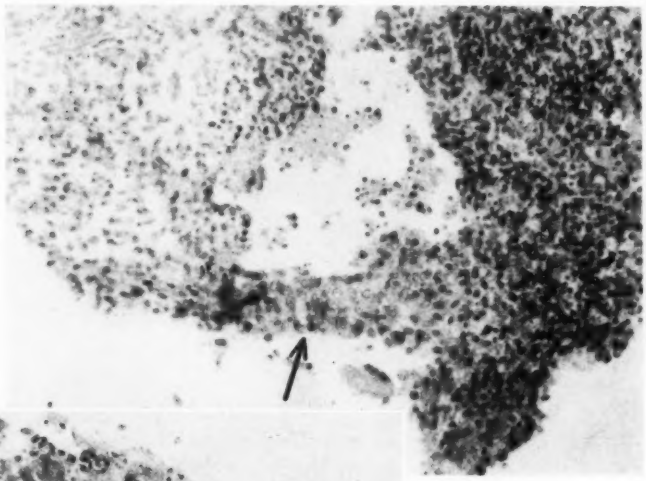
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Inoculated fetal horse spleen cultured for 8 days by the flask method, fixed in Zenker-acetic acid and stained with hematoxylin and eosin. The arrow points to a collection of plump reticulo-endothelium-like cells. Absence of necrosis and good state of preservation of the tissue may be noted. Inclusions are present, but the magnification is too low for adequate demonstration. × 200.

FIG. 2. Inoculated fetal horse lung cultured for 7 days by the flask method, fixed in Zenker-acetic acid and stained with hematoxylin and eosin. Structural detail is clear and the tissue is well preserved. Only a few pyknotic nuclei are seen. Inclusions can be seen, but the magnification is too low for good definition. The large inclusion seen in this field is shown in Figure 3. × 200.

FIG. 3. Photomicrograph of the lung (Fig. 2). The arrows indicate several distinct inclusions, one of which is exceptionally large. In the cell in the lower portion of the field a nucleolus is sharply stained and can be compared to the inclusion bodies. Oil immersion. × 1425.



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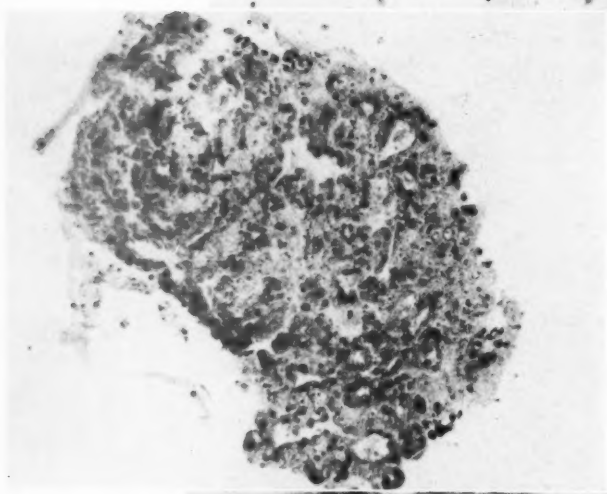

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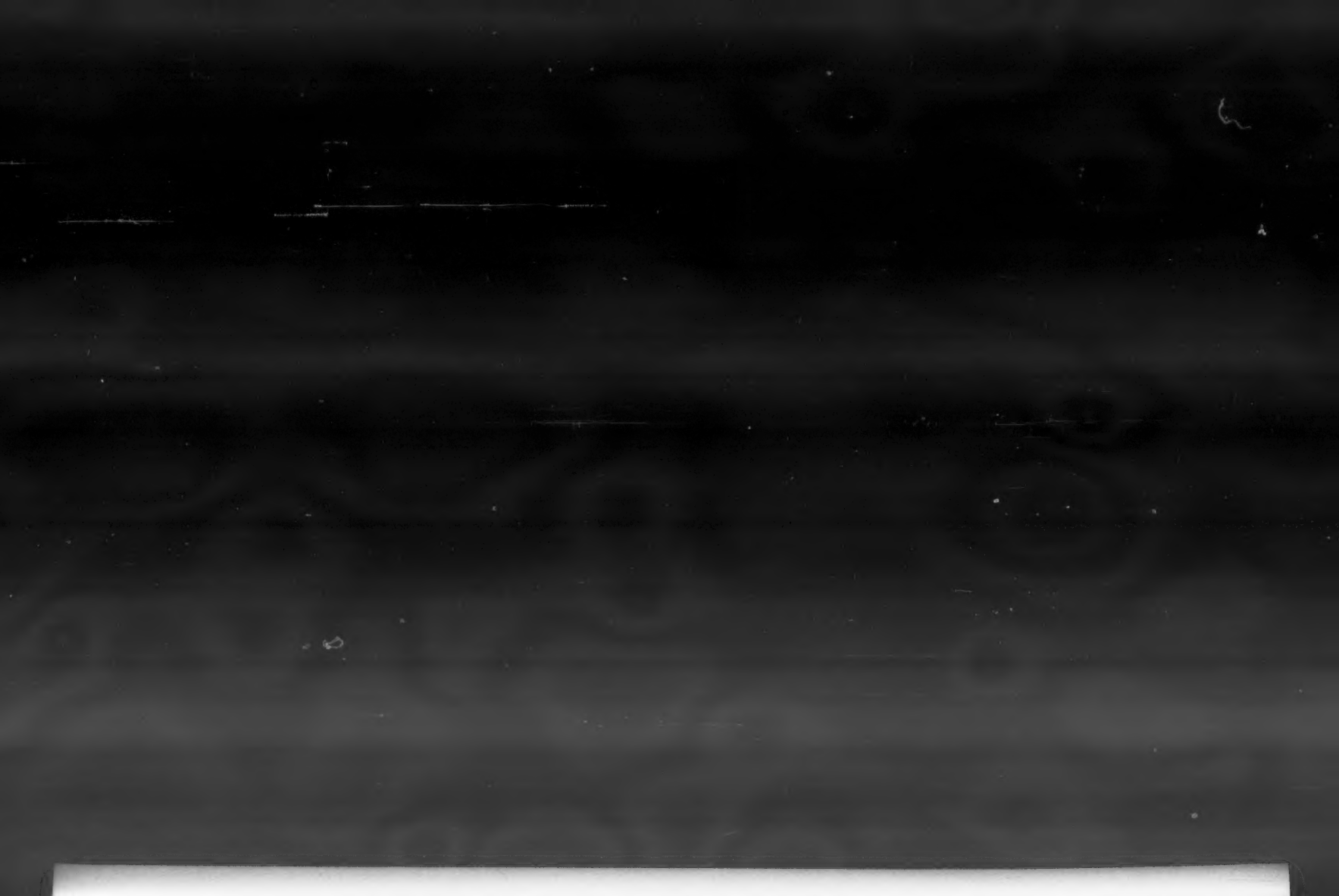
FIG. 4. Fetal horse spleen, ninth serial virus passage, cultured for 6 days, fixed in Zenker-acetic acid and stained with hematoxylin and eosin. Several inclusions are in focus; others are present but are not clearly shown. Oil immersion. × 1450.

FIG. 5. Fetal horse liver fixed in Zenker-acetic acid and stained with hematoxylin and eosin. The fetus was aborted by mare 249 (Table I), 14 days after receiving eighth passage, tissue-culture virus. An area of focal necrosis occupies the center of the field. × 75.

FIG. 6. Fetal horse spleen from the same source as Figure 5, fixed and stained by the same method. One inclusion in the center of the field is in sharp focus. Others are apparent, but are not clearly defined. × 1350.



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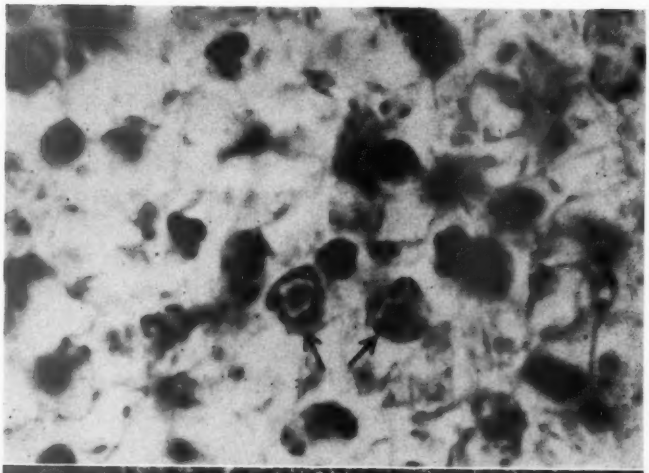

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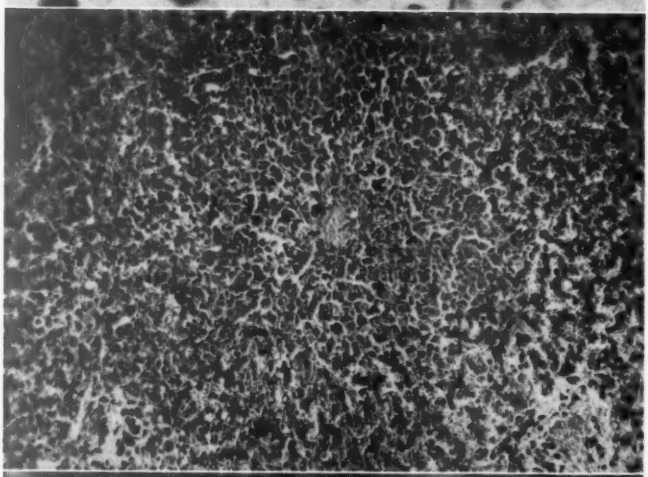

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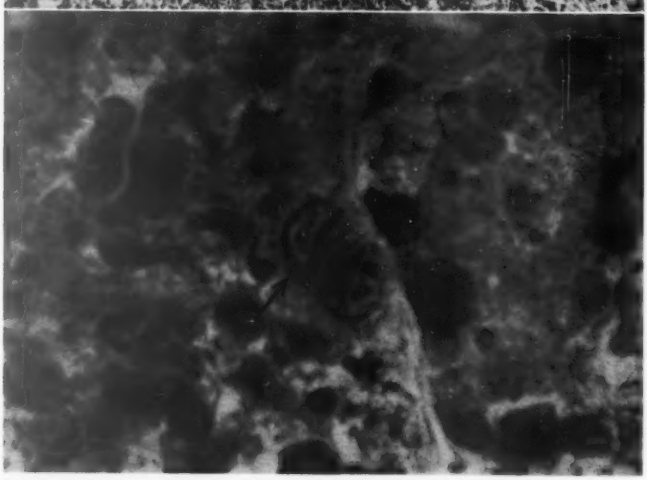

PSITTACOTIC LESIONS AND RESPIRATORY IMMUNITY IN INTRADERMALLY VACCINATED GUINEA-PIGS *

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The development of an effective method for vaccination of personnel against viruses of the psittacosis group has long been a baffling problem. Intraperitoneal injection of vaccines prepared from either phenolized or ultraviolet-irradiated virus induced resistance to intraperitoneal inoculation of infectious material,[1-8] but not against a respiratory exposure.[9] Following discovery of the virus,[10-12] Rivers and Berry[13] reported that rabbits became immune after recovery from either intracerebral, intratracheal, or intradermal injections of the virus. In 1934, Rivers and Schwentker[6] immunized monkeys against intratracheal inoculation by previous intramuscular injection of virulent agent. These authors reported that although intramuscular injection of living psittacosis virus was attended with no symptoms or signs of disease, neutralizing antibodies appeared in the blood of both man and monkeys.

By means of previous intradermal injection of different strains of psittacosis virus, attempts were made to produce resistance in guinea-pigs to an intratracheal inoculation with living psittacosis virus. The mechanism of this resistance was sought in a study of both the distribution of the virus and the pathologic changes resulting from intradermal infection with the agent.

MATERIALS AND METHODS

Virus Strains. The following is a description of the nomenclature and origin of each of the strains used in these studies.

1. Strain Cal 10, isolated in ferrets from human throat washings by Francis and Magill,[14] was furnished by Dr. M. D. Eaton.

2. Strain S-F, isolated in 1940 from human cases by Eaton, Beck, and Pearson,[15] was also furnished by Dr. Eaton.

3. Psittacosis strain 6 BC was originally procured from Dr. K. F. Meyer.

4. The Borg strain was isolated by Olson and Larson[16] from an epidemic of severe pneumonitis in the Bayou region of Louisiana, and was supplied by Dr. N. H. Topping.

5. Mouse pneumonitis (Grebb) virus was obtained from Dr. Clara Nigg, who isolated it from apparently normal mice.

Cultivation of the Virus. Groups of chick embryos, incubated be-

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tween 6 and 7 days, were inoculated by the yolk sac routes with 0.25 ml. of virus suspension in concentrations usually killing embryos in 3 to 8 days. Impression smears for elementary bodies were made from the yolk sacs of sample dead embryos to confirm the specificity of the deaths. Infected yolk sacs were prepared from each strain as a 10 per cent suspension in nutrient broth, shell frozen in ampoules, and stored at —50° C. as a source of stock seed virus.

Titration of Virus. Titration for infectivity of each strain was carried out by inoculation with 0.25 ml. of decimal dilutions in nutrient broth into the yolk sac of normal chick embryos. The LD_{50} from the resulting death pattern was computed by the method of Reed and Muench.[17]

Morphologic Studies. The tissues were derived from the same guinea-pigs in which the distribution of psittacosis virus was determined after the intradermal injection of strain 6 BC. Tissues studied included the dermal lesion and its regional lymph nodes of the right axillary group, control lymph nodes from the left axillary, inguinal, or cervical regions, lung, spleen, liver, kidney, heart, and adrenal glands. Portions of each tissue were fixed in acetone, Helly's fluid, and in a mixture of picric acid, absolute alcohol, and 10 per cent formalin. All tissues were stained with Giemsa's stain modified by Lillie.[18] In the search for elementary bodies additional stains such as those of Nobel[8] and Nyka[19] were performed.

Since pathologic changes in skin and other organs followed intradermal injection of the infectious psittacosis virus and its culture medium of chick embryo yolk sac, it was necessary to define the rôle of the medium in the pathogenesis of these lesions. Therefore, the medium and virus inactivated with 0.2 per cent phenol was injected intradermally into one group and a suspension of fresh normal yolk sac of chick embryos of the same age and concentration as either the infective or the phenolized preparations, into another group.

Results

Active Immunity and Isolation Studies in Guinea-Pigs

In previous studies on active cross-immunity using psittacosis virus,[9] no significant respiratory resistance was induced in mice by subcutaneous inoculation of living virus. However, respiratory resistance to the Borg virus could be stimulated in guinea-pigs by intradermal vaccination with the living virus of the 6 BC strain, agreeing with the report of Rivers and Schwentker.[6] Therefore, attempts were made to see whether other strains of the psittacosis-lymphogranuloma venereum group induced similar resistance to the Borg virus.

Groups of guinea-pigs were inoculated intradermally with virus of strains S-F, 6 BC, Borg, Cal 10, and Grebb respectively. The approximate number of egg LD_{50} doses of virus inoculated for each strain is shown in Table I. Two weeks later, animals from each treated group

TABLE I

Respiratory Immunity in Guinea-Pigs to Living Psittacosis Virus

Immunization			Results of intratracheal inoculation with Borg virus	
Virus strain	Egg LD_{50} inoculated intradermally	Pulmonary virus 2 weeks later positive/negative	4 LD_{50} dead/total	40 LD_{50} dead/total
S-F-70	1.0×10^{6}	0/2	0/5	0/5
	1.0×10^{4}	0/2	0/5	1/5
6 BC-SW	2.0×10^{6}	0/2	0/5	0/5
	2.0×10^{4}	0/2	0/5	0/5
Borg 40w	6.4×10^{5}	1/2	0/2	0/3
	6.4×10^{3}	0/2	0/2	0/5
Cal 10-69	3.2×10^{5}	0/2	0/5	1/5
	3.2×10^{3}	0/2	0/5	1/4
Grebb	2.0×10^{8}	0/2	2/4	3/5
	2.0×10^{1}	0/2	1/5	3/5
Controls			4/5	4/4

were injected intratracheally with either 4 LD_{50} or 40 LD_{50} guinea-pig respiratory doses of Borg virus. At that time, 2 animals from each vaccinated group were tested also for the presence of living virus in their lungs. The results of these studies are presented in Table I.

In general, guinea-pigs injected intradermally with strains SF, 6 BC, Borg, and Cal 10, survived intratracheal infections with 4 LD_{50} of the Borg strain; two of the three fatalities occurred in the group vaccinated with Cal 10 strain, less virulent than the other strains. On the other hand, animals inoculated with mouse pneumonitis (Grebb) virus showed no significant respiratory resistance.

Since intradermal injection of various strains of psittacosis virus induced resistance to respiratory infection, the lungs were tested for the immunizing virus. Live virus was recovered from the lungs of only one of 20 vaccinated guinea-pigs, 2 weeks after intradermal vaccination (Table I). This exceptional animal had been vaccinated with the larger dose of virulent Borg virus. These data indicated that the "interference phenomenon" was not responsible for this induced respiratory resistance.

Distribution of Psittacosis Virus in Guinea-Pigs Following Intradermal Injection with Living Virus (6 BC)

The distribution of virus was studied in the tissues and organs of the animal during the immunization period. Guinea-pigs weighing 300 to 400 gm. were injected intradermally with 0.5 ml. of living psitta-

cosis virus, 6 BC strain, in a dose of approximately $3 \times 10^{5.0}$ egg LD_{50}. Inoculations were made in the shaven skin over the thorax, about 2 cm. from the shoulder in the right axillary line. At intervals of 1, 2, 3, 6, 9, 14, 21, and 35 days after vaccination, 3 animals were sacrificed and necropsied. Tissues examined morphologically and culturally for evidence of virus have been listed. Portions were weighed, ground, and emulsified as a 10 per cent suspension in plain nutrient broth, pH 7.2. To control contamination, the suspensions were treated overnight at 4° C. with a mixture of 0.5 per cent sodium sulfamerazine and 0.5 per cent sodium sulfasuxadine. Previous tests showed that these concentrations of mixed drugs did not influence the infectivity of the virus at 4° C.

Living virus in these emulsions was demonstrated by the response of 10 mice to intracerebral inoculation with 0.03 ml. of the test inoculum. Animals were observed for typical symptoms of infection and all, whether dying or surviving 21 days, were examined microscopically for viral elementary bodies. Table II records the tissue distribution of the agent as well as the essential morphologic changes at various intervals after injection.

After inoculation, virus was recovered regularly from the dermal site of injection and irregularly from the livers for the first 6 days. Virus was recovered from the spleen in only one case on the first day, and from the regional lymph nodes for 14 days after intradermal inoculation. At no time was psittacosis virus recovered from the heart, lungs, adrenal glands, kidney, blood, or distant lymph nodes such as cervical, inguinal, or left axillary.

Since virus was recovered specifically from the regional axillary lymph nodes, it is obvious that the agent reached these nodes through lymphatic drainage from the site of injection. However, occasional appearance of the agent in both spleen and liver indicated dissemination throughout the blood stream.

Morphologic Changes in Tissues of Guinea-Pigs Injected Intradermally with 3×10^5 Egg LD_{50} of Strain 6 BC

The essential changes were found at the site of injection and in the regional lymph nodes and liver.

Site of Injection. One day after the intradermal injection of psittacosis virus, a palpable, red, swollen area appeared at the site of injection. The size of the skin lesions is recorded in Table II. Maximal size was attained by the third day and then the area of swelling decreased. At the end of a week, a small brown crust, about 5 to 8 mm. in diameter, appeared in the center of the nodule. At 9 to 14 days,

TABLE II

Pathologic Effects and Virus Distribution after Intradermal Injection in Guinea-Pigs of Infectious Psittacosis Virus in Chick Embryo Yolk Sac

Interval after injection	Skin lesion		Right axillary lymph node		Spleen	Lung		Liver		Heart	
	Diameter	Virus	Size	Virus	Virus	Lesion	Virus	Lesion	Virus	Lesion	Virus
days	*mm.*										
1	10	+	Increased	+	o	–	o	–	o	–	o
	7	+	Increased	o	+	–	o	–	+	Myocarditis	o
	15	+	Increased	+	o	–	o	–	o	–	o
2	15	+	Increased	o	o	–	o	Focal necrosis	o	–	o
	20	+	Hemorrhage	+	o	–	o	–	o	Myocarditis	o
	12	+	Increased	+	o	–	o	–	+	–	o
3	25	o	Increased	+	o	–	o	–	+	–	o
	30	+	Increased	+	o	–	o	Focal necrosis	o	–	o
	25	+	2×	+	o	–	o	Focal necrosis	o	–	o
6	6	o	10×	+	o	Pneumonia	o	–	+	–	o
	4	+	5×	+	o	–	o	–	o	–	o
	2	+	10×	+	o	–	o	–	+	–	o
9	8 Depilated	o	15×	+	o	–	o	–	o	–	o
	8 Granuloma	o	15×	+	o	–	o	Focal necrosis	o	–	o
	8 Ceroid	o	15×	+	o	–	o	–	o	Endocarditis	o
14	5 Ceroid	o	10×	+	o	–	o	–	o	–	o
	6 Ceroid	o	15×	+	o	–	o	Focal necrosis	o	–	o
	8 Ceroid	o	15×	+	o	–	o	–	o	–	o
21	4 Ceroid	o	5×, granuloma	o	o	–	o	Granuloma	o	–	o
	4 Ceroid	o	3×	o	o	–	o	Granuloma	o	–	o
	5 Ceroid	o	2×	o	o	–	o	Granuloma, ceroid	o	–	o
35	3 Ceroid	o	2×, ceroid	o	o	–	o	–	o	–	o
	2 Ceroid	o	2×	o	o	–	o	–	o	–	o
	3 Ceroid	o	2×, ceroid	o	o	–	o	Granuloma, ceroid	o	–	o

the hair about the injection point disappeared; the brown center of the lesion was circular and depressed about 0.5 to 1.0 mm. beneath the surface. This pitted brown area persisted throughout 5 weeks of observation but its diameter decreased to 2 to 3 mm. The subcutaneous tissue, within a day after injection, showed intense engorgement of capillaries and veins with radiation of the congested vessels becoming maximum at 3 days and associated with considerable edema in the subcutaneous fat. After the first week, congestion disappeared and the underlying subcutaneous tissue became faintly brown. This brownish area decreased in size, while its pigment increased and concentrated at the bottom of the cutaneous dimple.

The early stages of the lesion had no specific microscopic features during the first 3 days. Polymorphonuclear leukocytes, edema, and capillary congestion predominated at first and later were replaced by vacuolated mononuclear cells with basophilic granules and plasma cells often containing Russell fuchsinophil bodies. These changes extended through the corium and the subcutaneous and submuscular layers. Proliferating fibroblasts and mononuclear cells of the type described were present in the subcutaneous tissues 6 days after injection. Necrosis of the cutaneous muscle bundles and ulceration of the epidermis were evident at 6 days in one animal (Fig. 1). Fibrosis and giant cell formation by sarcolemmal nuclei had occurred about fibers which still revealed evidence of acidophilic coagulation necrosis. Infiltration by mononuclear and foreign body giant cells was found at the borders of these involved muscles. The necrotic elements of the exudate had coalesced to form small masses at the base of the ulcer, healing by newly proliferating cutaneous epithelial cells. Recently, Rustigian and Pappenheimer[20] found myositis in mice following intramuscular injection of viruses of the encephalomyelitis group and certain other neurotropic viruses. However, myositis due to psittacosis has not been described hitherto.

At 9 days, the injection site was still ulcerated. Multinucleated foreign body giant cells appeared in great numbers in the subcutaneous tissue. Their oval nuclei were clustered usually at one pole and oriented radially. Rarely histiocytes had mitotic nuclei. The cytoplasm of the histiocytes and giant cells was finely vacuolated. Occasionally, the relatively clear spaces in the cytoplasm had a yellowish hue with hematoxylin and eosin stains, or a blue-green color with the Giemsa stain. Frequently, these giant cells formed rims about large cyst-like spaces suggesting a reaction to necrotic fat (Fig. 2). These subcutaneous lesions resembled the lipoid granulomas of Abrikossoff,[21,22] Maggi,[23] Smetana and Bernhard,[24] and others. Fibroblastic prolifera-

tion was abundant throughout the infiltrating area. Capillaries with hyperplastic endothelium were often surrounded by mononuclear cells with dark basophilic cytoplasm. From 9 through 21 days, the number of giant cells with foamy vacuolated cytoplasm increased in the subcutaneous tissue (Fig. 3). The yellowish pigment, previously described, became abundant within these cells. This ceroid pigment[25-29] fluoresced in ultraviolet light and was stained by carbolfuchsin which resisted decolorization in hydrochloric acid. The pigment was also lipophilic, staining with Sudan III and Sudan black even after treatment with lipoid solvents such as alcohol and xylol. At the end of 5 weeks, the ceroid condensed within shrunken giant and mononuclear cells in the scarred subcutaneous tissues, accounting for the brown cutaneous dimple at the site of injection. The giant cells, so characteristic of the granulomatous lesions at the site of injection, did not appear until 9 days after injection, when the virus was no longer detectable in the dermal area of inflammation (Table II).

Lymph Node Changes. The increase in relative size of the regional right axillary lymph node as compared with the contralateral left axillary lymph nodes has been noted previously (Table II). Enlargement and hyperplasia had no specific features for the first 2 weeks. Mitotic nuclei were abundant in germinal follicles and within reticulum cells of the pulp. A large number of mononuclear cells with basophilic cytoplasm, resembling those seen in the skin, also occurred in dilated sinusoids of the lymph nodes. The thickened and adherent capsule at 6 days was due to fibroblastic proliferation and infiltrating mononuclear cells extending into adjacent fat (Fig. 5).

Specific structural changes appeared at 3 weeks, when the regional lymph nodes decreased in size. In the pericapsular fat, small granulomata of mononuclear and giant cells resembled those in the subcutaneous tissue (Fig. 6). By the fifth week, the cytoplasm of the giant cells had lost its delicate foamy character and contained globules and granules of intensely acid-fast ceroid pigment of various sizes. Similar pigment was seen in occasional mononuclear cells of the capsule or in the sinusoids of the pulp. As in the skin, the specific granulomatous change did not appear until after the virus was no longer detectable in the tissue.

Liver Changes. On the second day after intradermal injection of psittacosis virus, there was eosinophilic necrosis of liver cells and slight mononuclear cell infiltration about these focal areas in various parts of the lobule. Kupffer cells remained intact. Adjacent liver cells were finely vacuolated and pale-staining. Frequently, at the margins of the pale-staining areas, atrophic liver cells were more basophilic.

During the first 2 weeks, the necrotic areas increased in size. Sometimes healing occurred at 2 weeks after injection, with mononuclear cells replacing the necrotic foci. At 3 weeks, necrosis was no longer evident. However, focal accumulations of mononuclear and giant cells, resembling those seen in the skin and lymph nodes, were abundant (Fig. 7). Frequently the cells contained granules and globules of ceroid pigment which was demonstrated by the Kinyoun modification of the Ziehl-Neelsen stain.[28,29]

In the liver, the specific granulomatous lesions did not appear until the virus had disappeared or was no longer detectable. This series of events resembled that previously described in the skin and regional lymph nodes. The incidence of focal necrosis and detectable virus in the individual livers, as listed in Table II, indicated that in each instance focal necrosis was observed only in a liver in which virus was not detected. During the first 6 days, 3 of 12 livers showed focal necrosis and 5 others of the same 12 contained virus.

A search for elementary bodies was fruitless in the lesions of the skin, regional lymph nodes, and liver of these guinea-pigs when made with Giemsa, Nobel, and Nyka stains. Rivers and Berry[13] reported that tissues of guinea-pigs infected with psittacosis virus by intraperitoneal injection revealed no elementary bodies.

Changes in Other Tissues of Guinea-Pigs Injected with Psittacosis Virus. Virus was detected in one spleen on the day after the intradermal injection of virus. However, no specific morphologic changes were observed in this organ throughout the 5 weeks after injection. From the distribution of the virus 1 day after injection, it was apparent that it was disseminated through the systemic circulation within a day after injection, although at no time during 5 weeks after intradermal injection was it demonstrable in the blood.

Myocardial or endocardial inflammation occurred in 3 of 24 guinea-pigs of this series, resembling that described by others. These lesions were not confined to the guinea-pigs which received the infectious virus, appearing in control groups injected with normal yolk sac. Virus was not detected in the heart.

Interstitial pneumonia, with rare foci of interstitial infiltration with fibrin and polymorphonuclear and mononuclear leukocytes, was seen in the lungs of one guinea-pig 6 days after intradermal injection of virus. The absence of virus in the lungs and of elementary bodies in the pneumonic areas makes it difficult to attribute this lesion to the virus. A similar pulmonary lesion occurred in one of 14 guinea-pigs injected with normal yolk sac (Table III).

TABLE III

Pathologic Effects of Intradermal Injection of Normal Yolk Sac in Guinea-Pigs

Interval after injection	Skin lesion size	Right axillary lymph node	Spleen	Lung	Liver	Kidney	Heart
days	*mm.*						
1	0	Congested	—	—	—	—	—
	10	Increased	—	—	—	—	—
2	4	—	—	—	—	—	Myocarditis
	4	—	—	—	—	Pyelonephritis	—
3	0	2×	—	—	—	—	Myocarditis
	0	3×	—	—	—	—	—
7	0	3×	—	Pneumonia	—	—	—
	0	5×	—	—	—	—	—
9	0	3×	—	—	—	—	—
	0	2×	—	—	—	—	—
14	0	2×	—	—	—	—	—
	0	4× Abscess	—	—	—	—	—
22	0	—	—	—	—	—	Endocarditis
	0	—	—	—	—	—	—

TABLE IV

Pathologic Effects of Intradermal Injection in Guinea-Pigs of Phenolized Psittacosis Virus in Chick Embryo Yolk Sac

Interval after injection	Skin lesion size	Right axillary lymph node	Spleen	Lung	Liver	Kidney	Heart
days	*mm.*						
1	8		—		—	—	—
	10		—	—	—	—	—
2	0	3×	—	—	—	—	—
	4	2×	—	—	—	—	—
3	0	3×	—	—	—	—	—
	0	3×	—	—	—	—	—
7	0	10×	—	—	—	—	—
	0	2×	—	—	—	—	—
9	0	Increased	—	—	—	—	—
	0	8×	—	Lobular pneumonia	—	—	—
14	0	2×	—	—	—	—	—
	0	4×	—	—	—	—	—

Control Experiments with Normal Yolk Sac and with Phenolized 6 BC Strain of Psittacosis Virus in Chick Embryo Yolk Sac

Control experiments, in which normal yolk sac or phenolized 6 BC strain of psittacosis virus in yolk sac were employed, revealed early

changes similar to those seen after injection of infectious material. However, these changes were less severe and of shorter duration in the skin and regional lymph nodes and are summarized in Tables III and IV, respectively.

Skin lesions in both of the control series were similar. One day following intradermal injection, the area of swelling and redness was maximal, regressing to about half size at 2 days and disappearing at 3 days; microscopically, there were subcutaneous infiltration with polymorphonuclear and mononuclear leukocytes, edema, and capillary dilatation. In 2 days considerable infiltration by mast cells in the corium and by mononuclear and few polymorphonuclear leukocytes in the subcutaneous fat was evident, with very little capillary engorgement or edema. At 3 days, a few mononuclear cells with foamy cytoplasm were found in subcutaneous fat. Thereafter, for the 22-day period of observation, no lesions were found at the site of injection.

Lymph node changes were greater in the group injected with phenolized virus and yolk sac than with normal yolk sac. Neither capsular fibrosis, granulomata, nor ceroid appeared in these regional lymph nodes. Fourteen days after injection of normal yolk sac, the regional lymph node of one guinea-pig contained an abscess infected with Gram-positive cocci.

Hepatic lesions did not occur in any animal of either control series. However, the incidental lesions (Tables III and IV) resembled those seen in the live virus group (Table II).

These control observations indicated that the significant lesions which followed intradermal injection of the infectious 6 BC strain of psittacosis virus in yolk sac were at the site of injection, the regional lymph nodes, and the liver. The other lesions such as pneumonia, carditis, and pyelonephritis were unrelated to the injection of infectious virus. The skin lesions during the first 3 days could be attributed in part to trauma incident to the injection and to the presence of material derived from the chick embryo. Thereafter, the skin changes were related only to the infectious virus. To a considerable degree the lymph node alterations were modified by the products of the normal yolk sac as well as of the phenolized virus in yolk sac. These morphologic alterations were non-specific in character and in no way resembled the specific granulomatous changes seen in the regional lymph nodes of animals injected with the infectious virus and yolk sac. The granulomatous changes with ceroid formation were due, therefore, to no portion of the inoculum other than the infectious virus. Since hepatic lesions were absent in guinea-pigs of the control series, the

focal necrosis and granulomatous changes appearing after intradermal injection of infectious virus could be attributed only to the virus.

Discussion

The relationship of the production of ceroid pigment in reactions to infection has not been described. Recently Pappenheimer, Kunz, and Richardson[30] described ceroid in the pancreas and in splenic and intestinal lymphoid tissue of adult mice infected with Coxsackie virus (Connecticut S strain). Whether the ceroid was related to inflammatory changes of the infection or to the nutritional deficiency secondary to the pancreatic disease of the mice was not disclosed. However, the relation of vitamin E[27-29,31,32] and other nutritional deficiencies[25,29,33] to the occurrence of ceroid pigment has been well established. This pigment has been described in a variety of lesions in animals and man.[28,34] That vitamin E or any other nutritional deficiency played no rôle in the present studies was evident from certain features of the experiment. The guinea-pigs were on a natural food diet and showed no indication of any nutritional disturbance. No sign of pigmentation of uterus or testis or of muscular dystrophy was found. No atrophy of gonads was seen. The possibility that local infection with psittacosis virus caused local disturbances resembling those of vitamin E deficiency must be considered.

In spite of systemic effects of intradermal injection of psittacosis virus cultivated in yolk sac, no pulmonary involvement was detected either by inoculation of lung tissue into susceptible hosts or from morphologic evidence. Rivers *et al.*[12,35] and others[36] have pointed out that pulmonary lesions occurred only with intratracheal or intranasal instillation. The respiratory resistance to psittacosis which followed intradermal infection may be explained by the changes produced by the ensuing infection. Intradermal injection of psittacosis virus in guinea-pigs caused a specific systemic disease with a short period of fever and specific lesions at the site of inoculation, regional lymph nodes, and liver. The lesions persisted for at least 5 weeks after infection. Recovery from this form of psittacosis conferred immunity on the host. These findings confirmed the earlier observations of Rivers and Berry[13] and Rivers and Schwentker[6] that animals which had recovered from psittacosis were resistant to reinfection.

Summary and Conclusions

Guinea-pigs injected intradermally with psittacosis virus developed resistance to intratracheal infection. However, none of the animals of the present series which had been treated in this manner had detect-

able lesions or virus in the lungs at any time after the intradermal injection.

The intradermal injection, into guinea-pigs, of infectious psittacosis virus, strain 6 BC, cultivated in chick embryo yolk sac, produced a systemic disease with slight fever of 3 days' duration.

Virus was detectable, by mouse brain inoculation, in tissues from the site of injection for 6 days, in the regional lymph nodes for 14 days, and in the liver for 6 days. It was recovered from spleen 1 day after injection.

A specific granulomatous lesion with giant cells, histiocytes, and formation of ceroid pigment appeared at the site of injection, in the regional lymph nodes, and in the liver, when the virus was no longer detectable. These reactions of psittacosis conferred resistance to reinfection.

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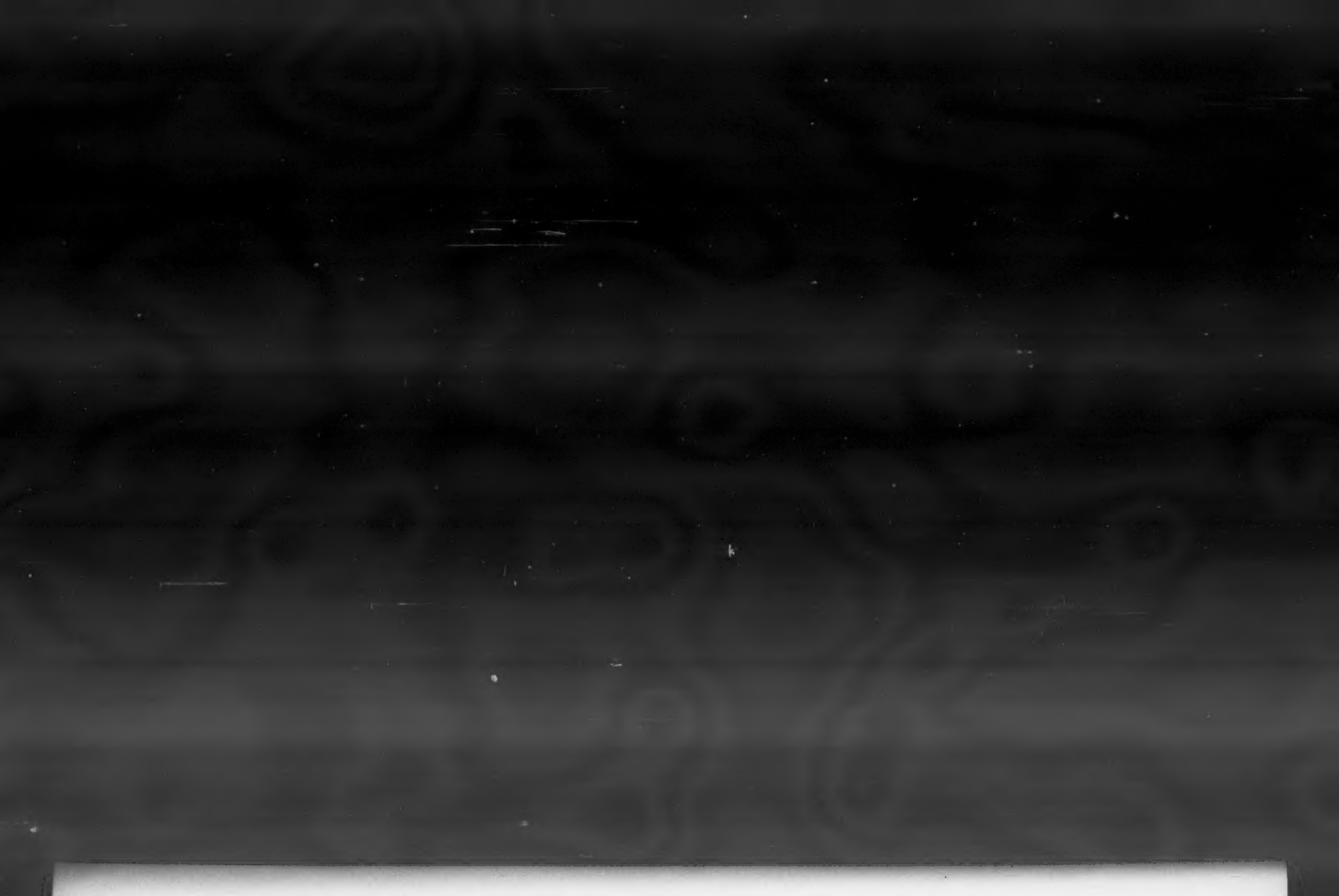
LEGENDS FOR FIGURES

FIG. 1. Subcutaneous muscle 6 days after infection. Muscle necrosis, fibrosis, mononuclear cell infiltration, giant cell formation. Giemsa's stain. × 136.

FIG. 2. Cutaneous fat 9 days after infection. Fatty cyst-like spaces surrounded by foamy mononuclear cells and giant cells. Mononuclear cell infiltration of connective tissue. Giemsa's stain. × 374.



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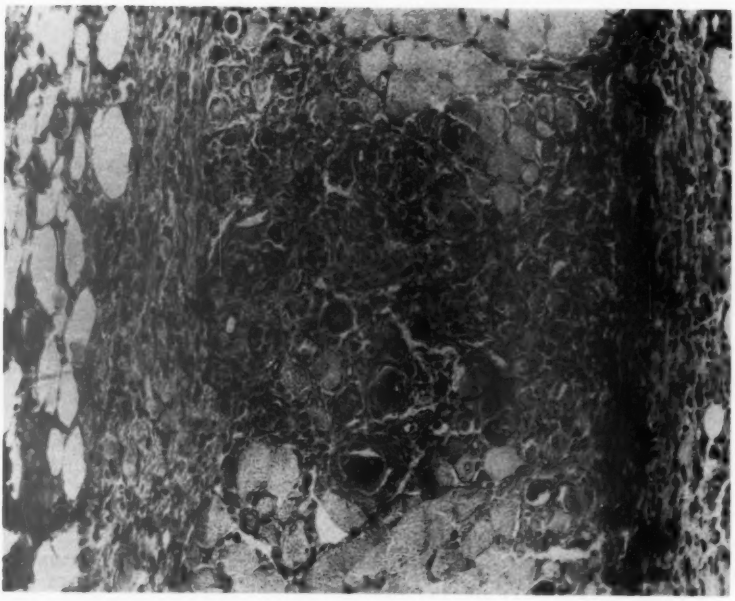

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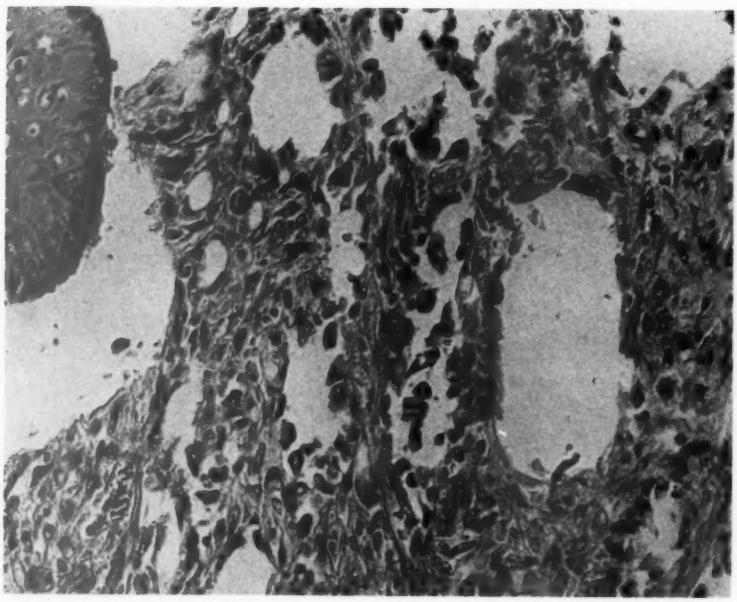
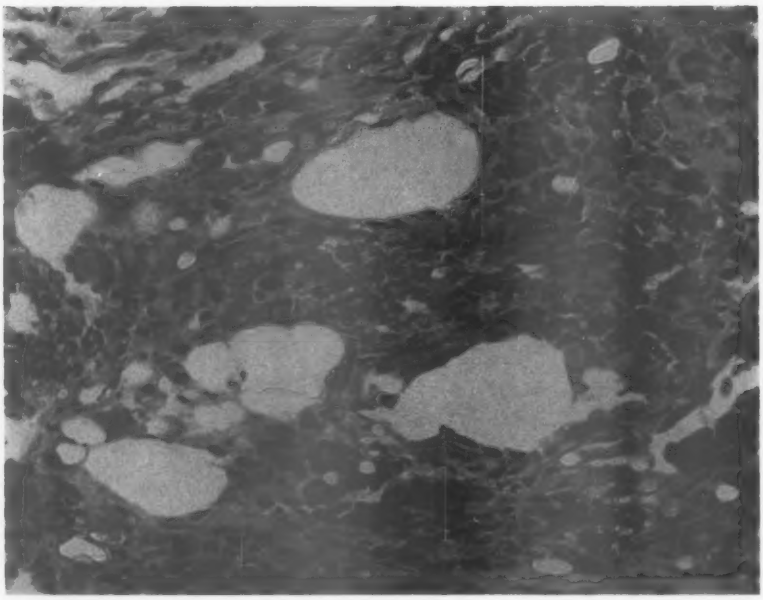


FIG. 3. Subcutaneous tissue 21 days after infection. Fat-containing cyst-like spaces, and cells containing ceroid lining the inner border of the spaces. Intense infiltration with mononuclear, foam, and giant cells. Giemsa's stain. × 374.

FIG. 4. Subcutaneous tissue 35 days after infection. Fat replaced by dense mononuclear and giant cells containing ceroid which is black in the photograph. Fatty cysts lined by mononuclear and giant cells. Kinyoun stain counterstained with hematoxylin. × 136.

FIG. 5. Regional lymph node capsule 6 days after subcutaneous infection. Capsular thickening by infiltrating mononuclear cells and fibroblastic proliferation. Giemsa's stain. × 374.



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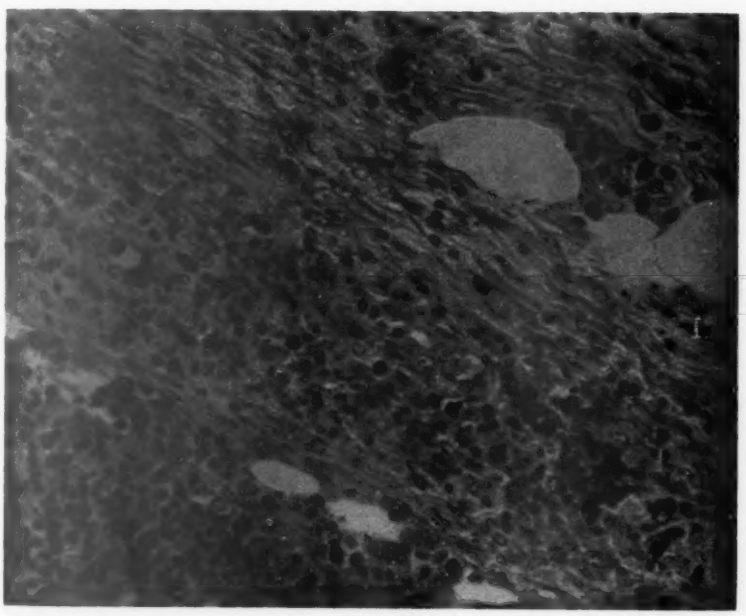
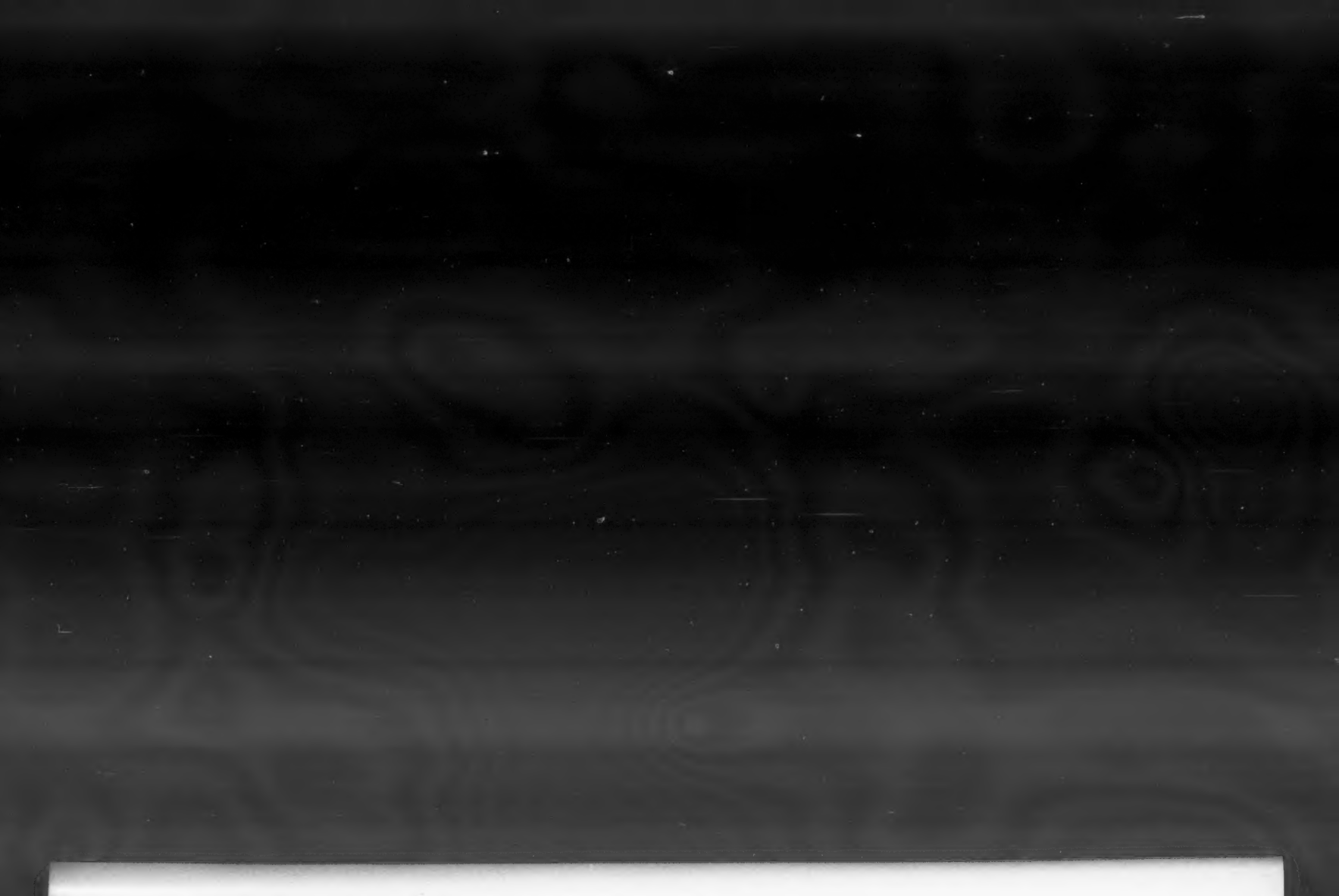


FIG. 6. Granuloma in pericapsular fat of regional lymph node 21 days after subcutaneous infection. Giemsa's stain. × 374.

FIG. 7. Granuloma with giant cell in liver 21 days after subcutaneous infection. This giant cell contained ceroid. Giemsa's stain. × 374.



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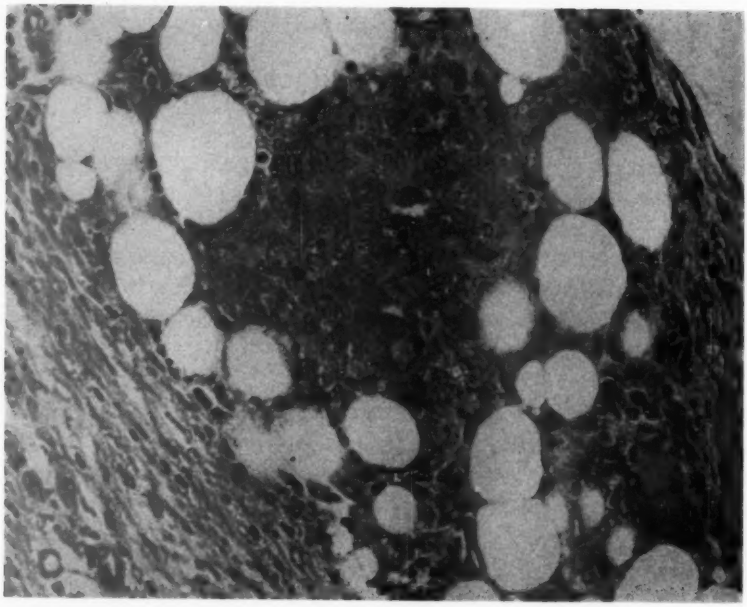

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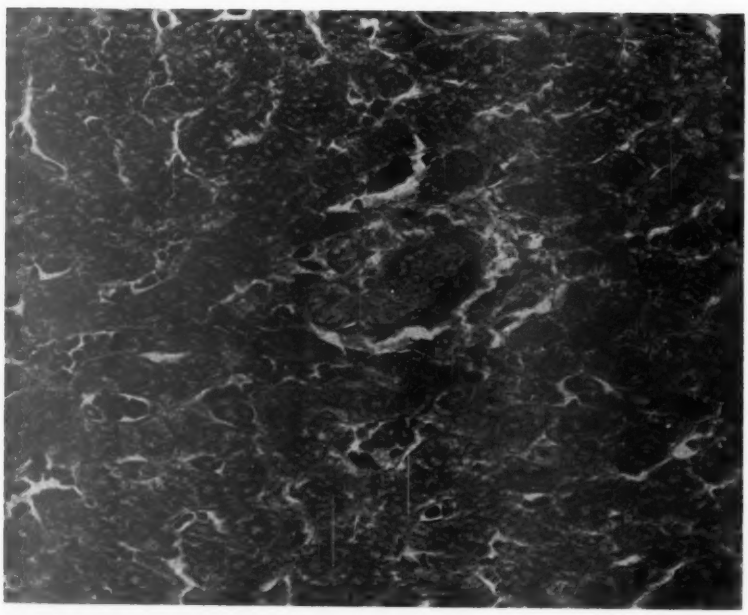

ELECTRON MICROGRAPHS OF ERYTHROCYTES FROM COTTON RATS INFECTED WITH THE LANSING STRAIN OF POLIOMYELITIS VIRUS *

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The Lansing strain of poliomyelitis virus[1] used for this study was furnished by Dr. David Bodian of the Poliomyelitis Research Center, Johns Hopkins University, Baltimore, Maryland. At that laboratory it had been passed 200 times in Swiss albino mice by the intracerebral route, and at our laboratory it was passed three additional times in cotton rats (*Sigmodon hispidus*) before initiating the present experiment.

MATERIALS AND METHODS

Thirty healthy cotton rats (age 14 days) were divided into two equal groups. One group was designated as the control group and the other as the test group. The brains from rats of the third intracerebral passage were ground with alundum and diluted to a 10 per cent suspension with physiologic saline solution. The virus in this passage titrated 10^{-2} intracerebrally in Swiss albino mice. Of this suspension, 0.5 ml. was injected intraperitoneally into each of 15 cotton rats of the test group. Animals of the control group were inoculated intraperitoneally with 0.5 ml. of a 10 per cent brain suspension prepared from normal cotton rats. The animals from each group were further divided into five groups of 3 animals each. Three animals from the test group were bled from the heart 2 hours after inoculation. Approximately 0.5 ml. of blood was drawn from each animal. A few drops of blood from each of the 3 animals were pooled in 1 ml. of citrated physiologic saline solution. The remaining blood was pooled and allowed to clot. The erythrocytes were washed three times by centrifugation and resuspension in physiologic saline solution and were then resuspended in 1 ml. of saline solution. The coagulated blood was centrifuged for 5 minutes at 2,000 r.p.m. and the serum removed. The cells and serum were then employed for electron microscopic studies. At the same time 3 animals from the control group were bled and their blood was subjected to the same treatment as described. The same procedure was carried out at 4, 10½, 19½, and 26 hours after inoculation. All animals from both groups which survived the initial bleeding were bled at 98 hours following inoculation, at which time a majority of the ani-

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mals of the test group showed involvement of the central nervous system.

Each specimen was prepared for electron microscopic examination by placing small drops of the material on parlodion film supports which had been prepared 48 hours previously. The films were dried and shadowed with chromium[2] at arc tangent 2/10 and examined under the RCA electron microscope, type EMU.

In order to determine the infectivity of the 12 specimens (6 blood cell suspensions and 6 sera) from the test group, the material was inoculated intracerebrally into Swiss albino mice. Seventy-two mice were divided into 12 groups of 6 mice each, and each group was inoculated with one specimen. In all cases 0.03 ml. of the material was used as the inoculum. In order to determine the presence of any material toxic for mice in normal cotton rat blood, each of 6 mice was inoculated intracerebrally with 0.03 ml. of an erythrocyte suspension from the 2-hour control group, and 6 mice were inoculated with 0.03 ml. of serum from the same group. All mice were observed twice daily for 30 days.

Results

Upon examination of the specimens under the electron microscope, no virus-like particles were observed in the erythrocytes from the control group. Virus-like particles were not seen in the sera of either the normal or infected cotton rats. Virus-like particles were seen in some of the erythrocyte suspensions from the infected cotton rats. An electron micrograph (Fig. 1) of the 2-hour specimen shows numerous virus-like particles associated with an erythrocyte. These particles resemble particles previously demonstrated by electron microscopy of the Lansing strain of poliomyelitis virus.[3]

Electron micrographs of the 4, 10½, and 19½ hour specimens revealed only a few virus-like particles as shown in Figures 2, 3, and 4. An occasional virus particle was seen in the 26 and 98 hour periods (Figs. 5 and 6).

Table I shows the infectivity of the specimens when injected intracerebrally into Swiss albino mice. Those mice succumbing to the disease developed symptoms of central nervous system involvement within 8 to 20 days after inoculation. The results indicate that the virus was present in the blood for 10½ hours after inoculation with the maximum concentration occurring at the 2-hour period. However, the virus apparently was present in low concentration since only a small number of mice succumbed.

All 12 mice inoculated with material from the control group remained normal throughout the 30-day observation period.

Discussion

From the results obtained in the experiment, it was apparent that virus was still present in the blood of infected cotton rats for at least 10½ hours after inoculation. In experimental work closely related to these findings, Smith[4] was able to demonstrate the presence of murine poliomyelitis virus in defibrinated blood of mice (6 to 8 weeks old) ½ to 5 hours after inoculation, in serum ½ to 7 hours after inoculation, and in the spleen ½ to 12 hours after inoculation. All mice were inoculated intraperitoneally with defibrinated blood from monkeys which had been injected intracerebrally 72 hours previously with murine polio-

Table I

Response of Swiss Albino Mice Injected with Erythrocytes and Sera from Cotton Rats Infected with the Lansing Strain of Poliomyelitis

Bleeding interval	Mice inoculated* with erythrocytes	Mice inoculated with serum	Minimum to maximum† incubation period
hours			*days*
2	1/6	3/6	8–14
4	1/6	0/6	14
10½	0/6	1/6	20
19½	0/6	0/6	
26	0/6	0/6	
98	0/6	0/6	

* Numerator denotes number of mice that died. Denominator denotes number of mice injected.

† Time elapsing between inoculation and onset of symptoms.

myelitis virus. Horstmann[5] was able to demonstrate the presence of poliomyelitis virus (Y-SK strain) in the blood of cynomolgus monkeys and chimpanzees between 4 and 5 days after oral infection with the virus.

Although several virus-like particles appeared on the surface of the erythrocyte 19½ hours after inoculation, only two virus-like particles were seen on the erythrocyte at the 10½-hour period. These were not as distinct as in the other photographs. The virus at this time interval was apparently not present in sufficient concentration to produce paralytic symptoms in the Swiss albino mouse. The mice may have had, however, an inapparent infection. The virus-like particles on the surface of erythrocytes and in the surrounding medium appeared to resemble in diameter and contour the Lansing strain of poliomyelitis virus.[3] Umbilicated particles found on red blood cells by others[6] do not correspond in shape or size with the virus particles illustrated in this study. However, electron micrographs, made by previous investigators,[7] of influenza virus particles adsorbed on the surface of chicken erythrocytes do resemble in shape, but not in size, the virus particles shown in this investigation.

The inability to detect virus-like particles in the infected serum examined by electron microscopy may have been due to their wide dispersion in the serum.

The fact that only a few mice showed paralytic symptoms after inoculation with infected cells and sera indicated that the concentration of virus was low, since the virus titrated only 10^{-2} intracerebrally in Swiss albino mice at the initiation of the experiment. Observation of the electron micrographs (Figs. 4, 5, and 6) for those bleeding intervals following the 10½-hour period revealed only a few virus-like particles. This decrease in the number of virus particles observed, associated with the fact that mice inoculated with these specimens failed to show evidence of poliomyelitis, indicates that the virus was gradually leaving the blood stream.

Summary

Studies were made by electron microscopy of erythrocytes and sera of cotton rats infected intraperitoneally with poliomyelitis virus (Lansing strain). Electron micrographs of erythrocytes obtained from infected cotton rats at 2, 4, 10½, 19½, 26, and 98 hours after inoculation revealed the presence of virus-like particles resembling those of the Lansing strain of poliomyelitis. The 2 and 4-hour specimens were infectious for Swiss albino mice inoculated intracerebrally.

No virus-like particles could be demonstrated in the infected sera by electron microscopy. However, sera from the 2 and 10½-hour bleedings, upon inoculation intracerebrally into Swiss albino mice, produced poliomyelitic symptoms in 4 of 12 animals.

Upon comparison of micrographs of erythrocytes from infected rats with those of erythrocytes from normal rats, it was assumed that the virus-like particles observed on erythrocytes from infected rats were poliomyelitis virus. Their shape and contour resembled the virus particles of the Lansing strain of poliomyelitis described in a previous paper.[3]

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Electron micrograph of an erythrocyte from a cotton rat 2 hours after injection with the Lansing strain of poliomyelitis virus. Shadowed with chromium at arc tangent 2/10. Virus-like particles are indicated by the arrows. × 18,000.

FIG. 2. Electron micrograph of an erythrocyte from a cotton rat 4 hours after injection with the Lansing strain of poliomyelitis virus. Shadowed with chromium at arc tangent 2/10. × 18,000.

FIG. 3. Electron micrograph of an erythrocyte from a cotton rat 10½ hours after injection with the Lansing strain of poliomyelitis virus. Shadowed with chromium at arc tangent 2/10. × 18,000.

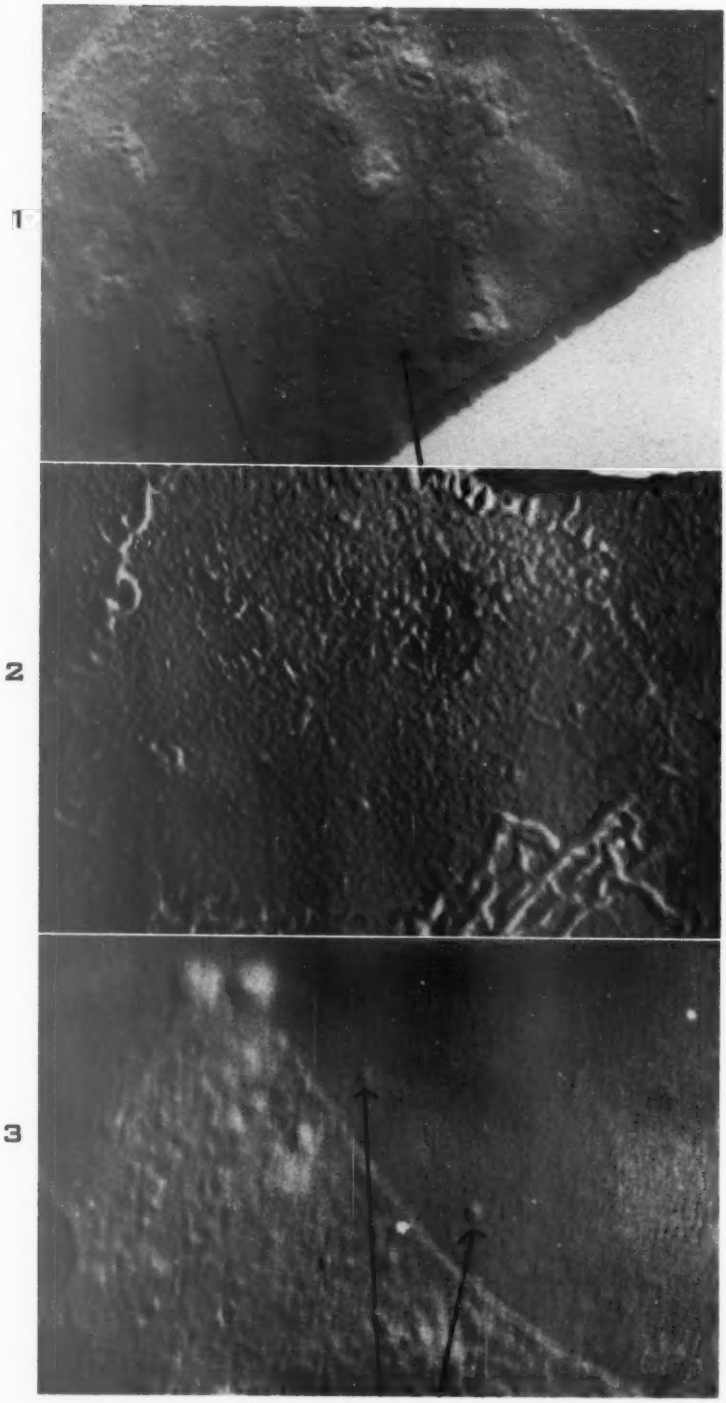
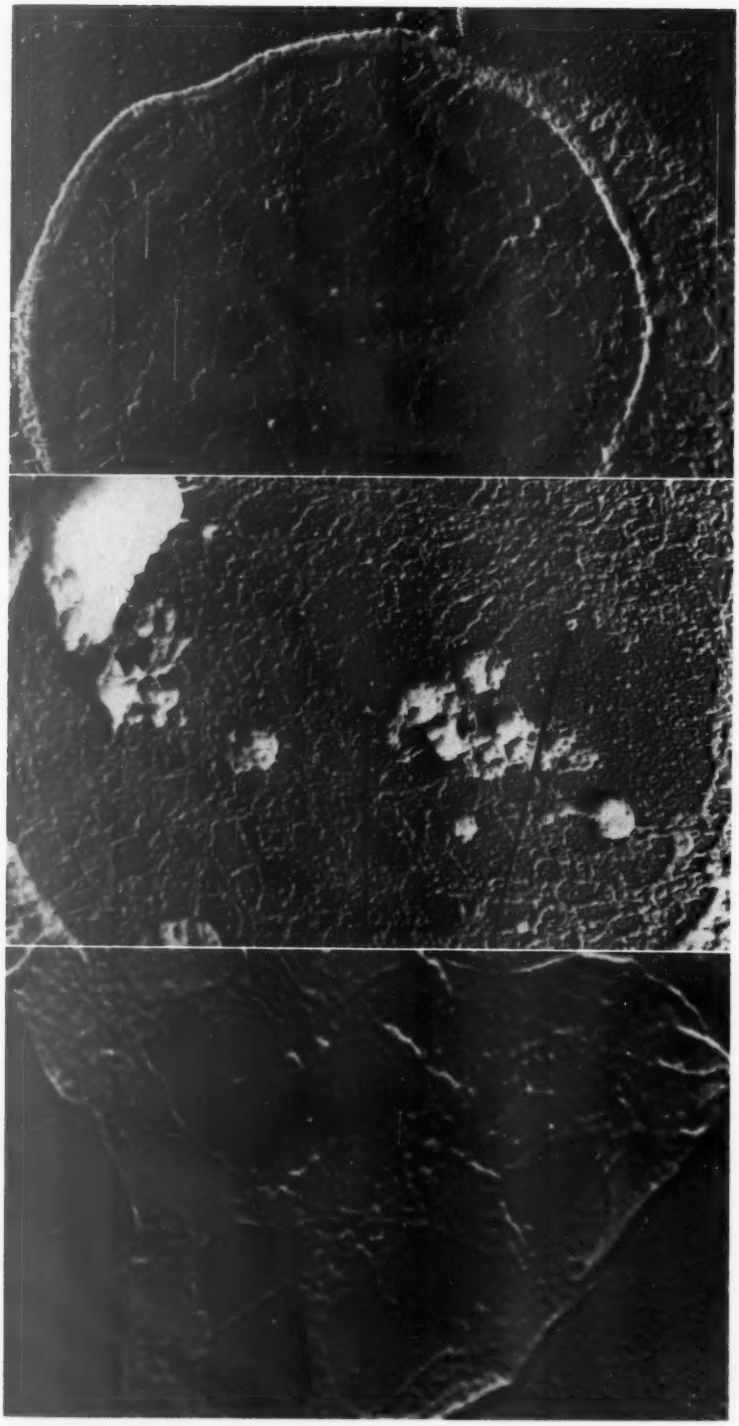


FIG. 4. Electron micrograph of an erythrocyte from a cotton rat 19½ hours after injection with the Lansing strain of poliomyelitis virus. Shadowed with chromium at arc tangent 2/10. × 18,000.

FIG. 5. Electron micrograph of an erythrocyte from a cotton rat 26 hours after injection with the Lansing strain of poliomyelitis virus. Shadowed with chromium at arc tangent 2/10. × 18,000.

FIG. 6. Electron micrograph of an erythrocyte from a cotton rat showing nervous symptoms 98 hours after injection with the Lansing strain of poliomyelitis virus. Shadowed with chromium at arc tangent 2/10. × 18,000.



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